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XXXIII. ON THE MECHANISM OF OXALIC ACID FORMATION BY *ASPERGILLUS NIGER.*

By HAROLD RAISTRICK and ANNE BARBARA CLARK.

From the Biochemical Laboratory, Cambridge.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

(*Received September 4th, 1919.*)

The production of organic acids, as a result of the growth of micro-organisms in solutions containing sugars, is of great importance both from a theoretical and practical point of view. A comparison of the acids produced by different organisms reveals the fact that, whereas the *bacteria* as a class chiefly produce monobasic acids, *e.g.*, acetic acid, butyric acid, and lactic acid, the formation of polybasic acids, *e.g.*, oxalic acid, citric acid, and fumaric acid, is almost entirely limited to the *fungi*. Even of the fungi the only family know to possess this chemical activity is that of the Aspergillaceæ. Although there were previously in the literature a number of isolated references to the production of oxalic acid by *Aspergillus niger*, the proof that this micro-organism really forms oxalic acid from sugar was not satisfactorily established until Wehmer published his classical researches on the subject in a series of papers in 1891. In addition to investigating the production of free oxalic acid from glucose under a variety of different conditions, he also showed that salts of oxalic acid are produced by the same fungus from salts of a few organic acids—notably from tartrates, malates, and citrates. He made no attempt, however, to offer any chemical explanation of the steps involved in the process. More recently Emmerling [1903] investigated the production of oxalates, by *A. niger*, from amino-acids and other protein derivatives. In particular, although he found that ammonium oxalate was produced in good yield from aspartic acid, he was quite unable to obtain any from the chemically allied substances—the ammonium salts of succinic, malic and tartaric acids. Heinze [1903], in a paper singularly devoid of experimental details, states that in the decomposition of glucose by *A. niger*, acetic acid is produced in addition to oxalic acid.

Until recently, it has been generally accepted that the production of citric acid from sugar was characteristic of a group of fungi, to which Wehmer gave the generic name of Citromyces, and that oxalic acid fermentation was almost equally characteristic of *Aspergillus niger*. That this is not so was proved by Currie [1917]. Working with a number of different strains of *A. niger*, he

showed that all of them produced from saccharose both oxalic acid and citric acid, the proportions of which could be varied by varying the conditions of the experiment. He pictured the general equation of metabolism of *A. niger* as follows:

Carbohydrate → citric acid → oxalic acid → carbon dioxide → mycelium,

and says "The equation has been written in this order, because *Aspergillus niger* will readily form oxalic acid when given only citric acid as a source of carbon."

A short time ago, Wehmer [1918] isolated another member of the Aspergillaceæ, to which he gave the name *Aspergillus fumaricus*, which produces from sugar fumaric acid, together with a little citric acid. These three polybasic acids, oxalic, citric, and fumaric, are not to be regarded as extraneous by-products of the degradation of sugar by these micro-organisms, as is, for example, the succinic acid produced during the alcoholic fermentation of sugar, which has been shown by Ehrlich [1909] to have its origin in the glutamic acid of the yeast protein. A consideration of the yield, which is in each case above 50 % of the sugar consumed, shows that these acids really represent definite stages in the degradation of the sugar molecule.

It will be noticed that, with the exception of Currie, no worker has attempted to offer any explanation as to the steps involved in the formation of these acids from sugar. The experiments in this paper were undertaken in the hope of elucidating the mechanism of the production of oxalic acid from sugar by *Aspergillus niger*. It is proposed to study the production of citric acid at some future time.

From a consideration of the literature, we came to the conclusion that a direct method of investigation, though admittedly more satisfactory, offered less hope of success than an indirect attack. The former method involves the isolation of intermediate products, in this case between sugar and oxalic acid, since it is certain that this action does not take place in one stage. The latter method involves the investigation of the production, or non-production, of oxalic acid from possible intermediate compounds, on the assumption that a failure to produce oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent a stage in the reactions involved in the production of oxalic acid. Production of oxalic acid, however, does not necessarily indicate that the substance in question is an intermediate product.

For these reasons, the method employed was to estimate the oxalic acid formed (but present as a salt of oxalic acid) when *A. niger* was cultivated in solutions containing salts of organic acids as the sole source of carbon. We were led to use salts of organic acids, rather than the free acids themselves, since, according to Wehmer, free oxalic acid, in the absence of other available carbon, is readily decomposed by *A. niger* at 37°, whereas fixation of the acid, as a salt, prevents its further decomposition by the fungus to any appreciable extent.

Experimental.

Throughout this work one strain of *Aspergillus niger* was used. This was obtained from Dr Paine of the Imperial College of Science and Technology, S. Kensington, and was identified by Mr F. T. Brooks of Cambridge. To both of them our thanks are due.

A culture solution was made up with the following composition:

Water	1000 cc.
NH_4NO_3	25 g.
KH_2PO_4	12·5 g.
$MgSO_4 7H_2O$	6·25 g.

20 cc. of this, diluted to 50 cc., gave one of Wehmer's solutions, having the following composition:

Water	50 cc.
NH_4NO_3	0·5 g.
KH_2PO_4	0·25 g.
$MgSO_4 7H_2O$	0·125 g.

This solution is subsequently referred to as "Culture Solution A." In some cases 1·5 g. of a salt of the acid under investigation was weighed out, dissolved in 30 cc. water, and to this 20 cc. of the undiluted culture solution was added. In other cases, where it was more convenient to use the free acid as a starting point, 1·5 g. of the acid was weighed out and neutralised with the necessary amount of normal alkali (*N* soda or *N* ammonia), as determined by titration. The volume was then made up to 30 cc. by adding the calculated amount of water from a burette, and, as before, 20 cc. of the culture solution was added. In each case the 50 cc. of solution was contained in a 200 cc. flask, autoclaved, inoculated with spores of *A. niger*, and incubated at 37° in the dark.

After a definite period of time, the oxalic acid content was estimated quantitatively by the usual method. The fungus was filtered off, and thoroughly extracted with hot dilute HCl, and hot water. The combined filtrate and washings were made distinctly alkaline with ammonia, and then acid with acetic acid. The oxalic acid was then precipitated from the boiling solution as the calcium salt, by the addition of calcium acetate and acetic acid. After standing overnight, the washed calcium oxalate was titrated with standard permanganate in the usual way.

This method was shown to be quantitative by adding a known weight of oxalic acid to 50 cc. of the culture solution A, and estimating as above.

	Oxalic acid added to culture solution	Oxalic acid estimated
(*a*)	0·1964 g.	0·1967 g.
(*b*)	0·1954 g.	0·1944 g.

The only difficulty met was with culture solutions containing tartaric acid. On standing overnight, a crystalline precipitate, probably consisting of calcium racemate, was found to be mixed with the calcium oxalate. The mixed calcium salts were filtered off, washed with hot water, re-dissolved in hot *N* HCl, made

alkaline with ammonia, and then made acid with acetic acid. After the addition of a little calcium acetate, and while the solution was still hot, the calcium oxalate was filtered off, washed, and estimated in the usual way. The calcium racemate remains in solution, and though a very small amount of calcium oxalate may also remain dissolved in the hot solution, the method is sufficiently accurate for our purpose.

Reference to the results given later shows that we were unable, under any conditions, to obtain any oxalate formation from salts of lactic acid. It was thought possible that the method might not be strictly quantitative in the presence of lactic acid. That this is not so was proved in the following way. To the filtrate and washings from sodium lactate C (Table IV, p. 335), to which had been added calcium acetate in the usual way, without any precipitation of calcium oxalate, was added 100 cc. standard oxalic acid solution (= 15·20 cc. potassium permanganate solution). The precipitated calcium oxalate required 15·46 cc. permanganate solution.

Preliminary Experiments.

In order to obtain some idea of the activity of *A. niger*, the amount of oxalic acid produced from the ammonium salts of a series of acids was determined. (In the case of glyoxylic acid, the calcium salt was used.) The results are summarised in the following table:

Table I.

1·5 g. acid as ammonium salt. Culture Solution A.

Acid	Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Butyric	27	–	—	—
Succinic	24	+ + +	0·3764	16·45
Fumaric	25	+ + +	0·2671	11·48
Malic	25	+ + +	0·3477	17·26
Tartaric	26	+ +	0·0609	3·39
Propionic	27	–	—	—
Pyruvic	26	+ + +	0·0115	0·53
Lactic	26	+ + + +	0	0
Acetic	26	+	0·0201	0·93
Glycollic	26	+	0	0
Glyoxylic (Ca salt)	32	+	0	0
Culture solution A as control	32	trace	0	0

+ + + + indicates extremely heavy growth.
+ + + „ heavy growth.
+ + „ fairly good growth.
+ „ distinct growth.
– „ no growth.

The oxalic acid produced is calculated as anhydrous $C_2H_2O_4$. The theoretical yield is calculated on the assumption that all the carbon of the acid

investigated (determined by titration) is converted into oxalic acid, and our results are expressed as a percentage of this value.

These results indicate that the chemical constitution of the acids, given as a source of carbon to the fungus, is of primary importance for the production of oxalic acid. Thus, although the best growth was obtained with the three-carbon acids, pyruvic and lactic, the amount of oxalic acid produced was nil or negligible, whereas with the four-carbon dibasic acids—succinic, fumaric, malic and tartaric—good yields were obtained. Similarly with the two-carbon acids—acetic, glycollic and glyoxylic—although growth was approximately the same in each case, acetic acid was the only one from which any oxalic acid was produced. It will be noticed that, although *A. niger* grew slightly in the culture solution alone, there was no growth at all when the ammonium salt of propionic acid or butyric acid was present. This fact was also observed by Wehmer, who was unable to obtain any growth from these two acids, under any conditions. This was probably due to hydrolytic dissociation of the organic salts, with the consequent formation of a certain amount of the free acids, which, above a certain concentration, are known to have a deleterious effect on the fungus [Duclaux, 1889]. For this reason, in the subsequent work two other culture solutions were made up, containing, in place of KH_2PO_4, the one Na_2HPO_4, and the other Na_3PO_4. It was hoped that this would have a beneficial effect in fixing any oxalic acid that might be formed.

Main Series of Experiments.

In the following series of experiments, the conditions of which are similar to those of the preliminary series, three culture solutions were used:

1. *Culture Solution A* has already been described.

2. *Culture Solution B.* A stock solution was made such that 20 cc. diluted to 50 cc. (as with A) had the following composition:

Water	50 cc.
NH_4NO_3	0·5 g.
Na_2HPO_4	0·25 g.
K_2SO_4	0·125 g.
$MgCl_2$	0·005 g.

3. *Culture Solution C.* A stock solution was made such that, on dilution, (as with A and B) it had the following composition:

Water	50 cc.
NH_4NO_3	0·5 g.
Na_3PO_4	0·25 g.
K_2SO_4	0·125 g.
$MgCl_2$	0·005 g.

The production of oxalic acid (as oxalates) from a large series of acids, present as sodium, ammonium, or calcium salts, was investigated. The results, which are summarised in the following tables, are expressed as in Table I. We have also incorporated with them, for comparison, the results given in Table I.

Table II.

Four-carbon dibasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Succinic	Na	A	38	+ + +	1·0503	45·90
	,,	B	38	+ +	0·8113	35·46
	,,	C	38	+	0·4227	18·47
	NH_4	A	24	+ + +	0·3764	16·45
	,,	A	38	+ + +	0·6360	27·80
Fumaric	Na	A	38	+ + +	1·050	45·11
	,,	B	38	+ +	1·074	46·14
	,,	C	38	+ +	0·7910	34·00
	NH_4	A	25	+ + +	0·2671	11·48
Maleic	Na	A	34	+	0	0
	,,	B	34	+	0·0049	0·28
	,,	C	34	+	0·0105	0·62
	,,	A	38	+	0	0
	,,	B	38	+	0·0080	0·48
	,,	C	38	+	0·0333	1·97
Malic	Na	A	38	+ +	0·6493	32·22
	,,	B	38	+ +	0·5506	27·33
	,,	C	38	+ + +	0·951	47·2
	NH_4	A	25	+ + +	0·3477	17·26
	,,	A	38	+ +	0·3170	15·73
Tartaric	Na	A	38	+	0	0
	,,	B	38	+	0	0
	,,	C	38	trace	0	0
	NH_4	A	26	+ +	0·0609	3·39

Table III.

Four-carbon monobasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
Butyric	Na	A	38	–	—	—
	,,	B	38	+	0·0080	0·33
	,,	C	38	–	—	—
	NH_4	A	27	–	—	—
Isobutyric ...	Na	A	38	–	—	—
	,,	B	38	–	—	—
	,,	C	38	–	—	—
β-Hydroxybutyric	Na	A	38	–	—	—
	,,	B	38	–	—	—
	,,	C	38	–	—	—
Hydroxyisobutyric	Na	A	38	–	—	—
	,,	B	38	–	—	—
	,,	C	38	–	—	—

Table IV.

Three-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
Lactic	Na	A	38	+ + + +	0	0
	,,	B	38	+ + +	0	0
	,,	C	38	+ + +	0	0
	,,	A	7	+ +	0	0
	,,	B	7	+ +	0	0
	,,	C	7	+ +	0	0
	NH_4	A	26	+ + + +	0	0
	Ca	A	38	+ + + +	0	0
	,,	B	38	+ + +	0	0
	,,	C	38	+ + +	0	0
Pyruvic	Na	A	38	+ + +	0·0134	0·61
	,,	B	38	+ +	0·1628	7·44
	,,	C	38	+ +	0·1478	6·75
	NH_4	A	26	+ + +	0·0115	0·53
Glyceric	Na	A	31	+ + +	0	0
	,,	B	31	+ + +	0·0213	1·81
	,,	C	31	+ + +	0·0258	2·19
	,,	A	38	+ + +	0·0120	1·02
	,,	B	38	+ + +	0·0838	7·11
	,,	C	38	+ + +	0·0181	1·54
	NH_4	A	38	+ + +	0	0
	,,	B	38	+ + +	0	0
	,,	C	38	+ +	0	0
Malonic	Na	A	38	+	0	0
	,,	B	38	+	0	0
	,,	C	38	+	0	0
	Ca	A	38	–	—	—
	,,	B	38	–	—	—
	,,	C	38	–	—	—
Propionic ...	Na	A	38	–	—	—
	,,	B	38	+ +	0·0825	4·64
	,,	C	38	trace	—	—
	NH_4	A	27	–	—	—

Table V.

Two-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
Acetic	Na	A	38	+ + +	0·3869	38·98
	,,	B	38	+ + +	0·4534	45·70
	,,	C	38	+	0·0111	1·12
	,,	A	38	+ + +	0·4211	42·44
	,,	B	38	+ + +	0·4534	45·70
	,,	C	38	trace	—	—
	NH_4	A	26	+	0·0201	0·93
Glycollic	Na	A	38	+	0	0
	,,	B	38	+	0	0
	,,	C	38	+	0	0
Glycollic (a different sample of glycollic acid)	,,	A	38	+ +	0·0034	0·20
	,,	B	38	+ +	0·0045	0·27
	,,	C	38	+ +	0·0036	0·22
	NH_4	A	26	+	0	0
Glyoxylic ...	Ca	A	32	+	0	0

Table VI.

One-carbon acid.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
Formic	Na	A	38	++	0	0
	„	B	38	++	0	0
	„	C	38	+	0	0

Control cultures containing only culture solution A, B, or C were inoculated with *A. niger* and grown for 38 days at 37°. No oxalic acid was formed.

In each experiment a control flask, containing the salt of the acid under investigation and culture solution, was incubated without inoculation. In no case was any oxalic acid formed.

Influence of the source of nitrogen on oxalate formation.

In the literature on oxalic acid formation from sugar by *A. niger*, frequent references are made to the importance of the source of nitrogen supply. Thus Wehmer, in Lafar's handbook [1910], says "A decisive influence is also exerted by the inorganic bodies present, especially the source of nitrogen for the growing fungus, the liberation of acid (oxalic) being absent (in spite of good growth), when ammonium chloride or sulphate is substituted for potassium, calcium, or ammonium nitrate (even in the presence of sugar),—and, in fact, these additions will prevent the formation of acid in cultures that would otherwise acidify at once." This he explains as follows: The assimilation of nitrogen from a nitrate by the fungus results in the liberation of a base from the nitrate, *i.e.*, NH_4OH, NaOH, KOH or $Ca(OH)_2$, which would fix a portion of the oxalic acid formed, thus preventing its decomposition. The assimilation of nitrogen from ammonium chloride or sulphate, however, would result in the production of a mineral acid—HCl or H_2SO_4—which would tend to favour the decomposition of the oxalic acid, which is known to be much more easily decomposed by *A. niger* than are its salts. This explanation seems a little difficult to accept, in view of the following facts:

1. Only a relatively small amount of the nitrogen supplied is metabolised as protein, as we have proved.

2. Currie [1917] has shown that oxalic acid, along with citric acid, is produced from sugar in culture media made acid to P_H 3·4–3·5 with HCl.

It seemed to us desirable, because of the importance that we attached to acetic acid as a probable precursor of oxalic acid, to prove whether the nitrate ion has any specific importance in the production of oxalates (as distinct from free oxalic acid) from sodium acetate. With this end in view, *A. niger* was cultivated on solutions containing sodium acetate as the sole source of carbon, the nitrogen being supplied as ammonium nitrate, sulphate, chloride or phosphate.

Otherwise the conditions of the experiment were as before. The results given in Table VII are expressed in the same way as in the other tables.

Table VII.

1·5 g. *crystalline Na acetate* (contains 0·904 g. $C_2H_3O_2$ Na).

Ammonium salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
NH_4NO_3	A	38	+ + +	0·4211	42·44
	B	38	+ + +	0·4534	45·70
	C	38	trace	—	—
$(NH_4)H_2PO_4$	A 1	38	+ +	0·4204	42·37
	B 1	38	+ + +	0·4836	48·73
	C 1	38	+ + +	0·4873	49·11
NH_4Cl	A 2	38	+ + +	0·4067	40·98
	B 2	38	+ +	0·4739	47·77
	C 2	38	trace	—	—
$(NH_4)_2SO_4$	A 3	38	+ + +	0·4054	40·86
	B 3	38	+ +	0·4707	47·43
	C 3	38	trace	—	—

Culture Solutions:

A, B, C have the same composition as previously.

A 1, B 1, C 1 have the same composition as A, B, C, with the exception that NH_4NO_3 is replaced by the same weight of $(NH_4)H_2PO_4$.

A 2, B 2, C 2, NH_4NO_3 replaced by NH_4Cl.

A 3, B 3, C 3, NH_4NO_3 „ „ $(NH_4)_2SO_4$.

These remarkably concordant results show that the nitrate ion has no specific effect on oxalate formation, and confirm the previous observation that *A. niger* can oxidise acetic acid to oxalic acid very smoothly.

Discussion of results.

A consideration of the results obtained with the four-carbon dibasic acids shows that, in general, they give remarkably good yields of oxalic acid. In particular, this is especially true of succinic, fumaric, and malic acids. The failure of *A. niger* to produce any oxalic acid from the sodium salts of tartaric acid is probably due to the fact that the fungus only grew very slightly. The poor growth and poor yield of oxalic acid obtained with sodium maleate is in agreement with the observation made by Buchner [1892], who showed that *A. niger* could utilise fumaric acid, but not maleic acid, as a source of carbon. This he was led to expect from the fact that, whilst fumaric acid is a normal constituent of many plants, the isomeric maleic acid has not yet been found in nature. The small amount of growth we did obtain was probably a result of the molecular rearrangement of a portion of the sodium maleate, under the conditions of our experiment.

The outstanding feature of the experiments with the four-carbon monobasic acids is the almost entire absence of growth and failure to produce oxalic acid. The isolated case in which growth and oxalic acid formation was observed justifies the conclusion previously made as to the reason for the failure of the fungus to grow in a solution containing ammonium butyrate.

Most surprising and unexpected results were obtained from the series of acids containing three-carbon atoms. In almost all cases we obtained magnificent growths of the fungus, which were, on the whole, better than those obtained with the four-carbon dibasic acids. In spite of this, the yield of oxalic acid was either nil or very small. In particular was this the case with lactic acid, which is known to be an almost constant product of the *bacterial* degradation of sugar. A somewhat similar result was obtained by Wehmer [1891]. He cultivated *A. niger* on a solution identical with our culture solution A, and obtained the following results. In one experiment with free lactic acid he obtained no oxalic acid after 101 days; in two experiments with potassium lactate, after the same length of time, he observed the formation of 3·67 % of oxalic acid in the one case, and of no oxalic acid in the other. Finally with calcium lactate, in two experiments having an incubation period of 43 days, he obtained 0·76 % and 0·96 % respectively. That the oxalic acid is not first formed, and then decomposed, is indicated by our results, which proved its non-production after an incubation lasting only seven days.

A comparison of the results obtained with the two-carbon acids—acetic, glycollic, and glyoxylic—shows a sharp differentiation between acetic and the other two acids. With the exception of the one experiment with ammonium acetate, on which the fungus only grew very slightly, the yield of oxalic acid obtained from acetic acid was from 39 % to 49 %, whereas with glycollic and glyoxylic acids the yield was either nil or negligible. The behaviour of *A. niger* towards these three acids offers another example of the difference between biological and chemical reactions, for although it is a relatively simple matter to oxidise glycollic and glyoxylic acids to oxalic acid *in vitro*, it is difficult to obtain oxalic acid from acetic acid. The ease with which *A. niger* oxidises acetic acid seems to us to be a very significant fact.

No oxalic acid is produced from the one-carbon acid—formic acid—although the growth was relatively good. This suggests that oxalic acid formation is not a question of synthesis.

Three facts have been proved conclusively in the experiments described in this paper:

I. Acetic acid is the only one of the three possible two-carbon acids which is oxidised by *A. niger* to oxalic acid.

II. Taken as a class, the three-carbon acids are not oxidised to oxalic acid by the fungus to any appreciable extent.

III. The four-carbon dibasic acids are, as a class, smoothly oxidised to oxalic acid, whilst the four-carbon monobasic acids give no oxalic acid.

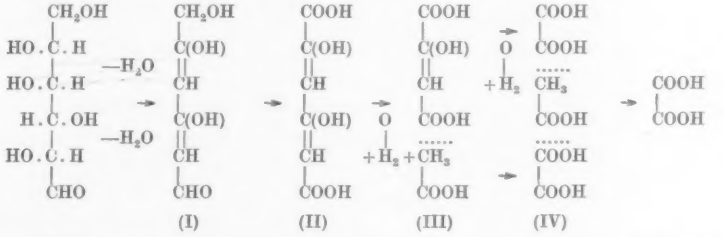
A consideration of these facts leads to the following conclusions:

(I) The breakdown of the sugar molecule by *A. niger* does not take place by a primary splitting of one molecule of sugar into two molecules of a three-carbon acid since, as was pointed out in the introduction, the non-production of oxalic acid from them, by *A. niger*, necessarily precludes the possibility of their being precursors of oxalic acid.

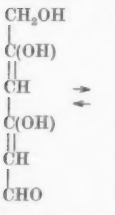
(II) The breakdown cannot take place carbon by carbon, leaving the last two carbon atoms in the chain to be oxidised to oxalic acid, since this would result in the production of one-carbon compounds, and it has been shown that *A. niger* is unable to *synthesise* oxalic acid. Also, if this hypothesis were correct, it would follow that the maximum yield obtainable would be about 33 %, whereas, in practise, the yield is usually about 50 %. It is also extremely unlikely on general grounds.

(III) The breakdown probably takes place in two stages, involving in the first stage the production, from one molecule of sugar, of one molecule of a two-carbon compound, and one molecule of a four-carbon compound. This is then broken down in the second stage into two molecules, each containing two carbon atoms.

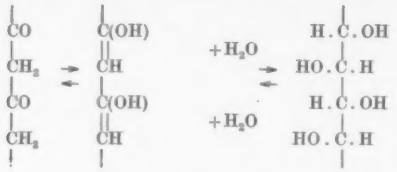
To meet these conclusions the following scheme is suggested, since it seems to fit in with all the known facts:



The first stage (I) suggested represents the formation, by simple dehydration, of the enol form of a "polyketide." So long ago as 1893 and, more recently, in 1907, Collie [1893, 1907], who proposed the term "polyketides" for the series of compounds containing $-CH_2 . CO-$ groups, pointed out the importance for biological chemistry of this class of compounds. These observations do not seem to us to have received from biochemists the consideration that they deserve. As a class, the "polyketides" give rise to a large series of compounds which either occur in nature or are closely related to naturally occurring compounds. Moreover, the reactions involved are of a very simple nature, *e.g.*, hydration, dehydration, polymerisation, etc., and very often take place at the ordinary temperature. In particular, Collie

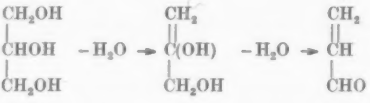


pointed out the close relationship between the sugars and the keten group—though at present we have no means of synthesising the sugars from keten or from any of its simple derivatives. Thus, if one takes two keten groups,

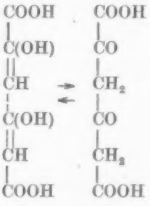


by simple hydration one arrives at a system of four-carbon atoms as present in the sugars.

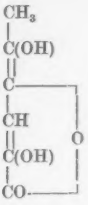
Conversely, the removal of two molecules of water from one molecule of sugar—a change which is probably very easily accomplished by the fungus—would immediately give rise to a very reactive compound. An interesting example of a similar type of change has recently been afforded by Voisenet [1918]. He isolated an organism, to which he gave the name *Bacillus amaracrylus*, which produces acraldehyde from glycerol. The changes involved are represented as follows:



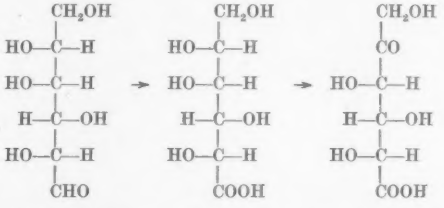
The second stage (II), involving the oxidation of the two terminal carbon atoms to carboxyl groups, is extremely probable in view of the oxidising powers which *A. niger* is known to possess. There is, of course, no evidence to show whether dehydration takes place before oxidation, or *vice versa*, but in either case there would result the formation of the enol form of a diketo-acid:



There are a number of examples of the production, by micro-organisms, of keto-acids from sugars—a fact which renders this change extremely probable. Traetta-Mosca [1914] isolated a fungus having the characteristics of *Aspergillus glaucus* which ferments sugars giving a hitherto unknown substance, which is in all probability the γ-lactone of a trihydroxyhexadienoic acid. For this acid, which is fermentable to alcohol by yeast, Traetta-Mosca suggests the following formula:



Also *Bacterium xylinum* [Adrian Brown, 1886] oxidises glucose first to gluconic acid, and then to keto-gluconic acid:



Weizmann's bacillus [1915], which produces acetone from the carbohydrates of maize, probably produces first aceto-acetic acid, which then loses CO_2 and forms acetone (Author's unpublished observations).

The subsequent breakdown of the diketo-acid II is readily explained. It contains a β-keto-linking, and (disregarding the δ-keto-linking for the moment) is thus a β-keto-acid. The β-keto-acids, as a class, are readily hydrolysed, giving an acid containing two carbon atoms less and acetic acid. This change is represented in stage III. The acetic acid is then oxidised to oxalic acid, a change which our results have shown to be easily accomplished by *A. niger*. The residual part of the molecule III is the enol form of the β-keto-acid—oxalacetic acid—which, undergoing a similar hydrolysis to II, gives rise to one molecule of oxalic acid and one molecule of acetic acid, which is then oxidised, as before, to oxalic acid.

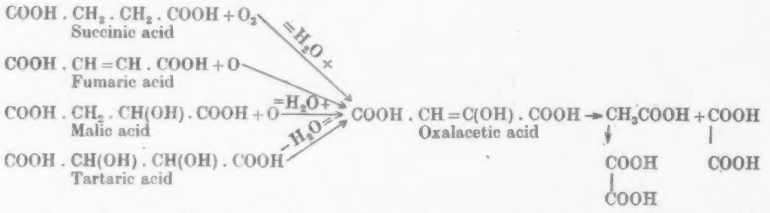
The scheme suggested involves the intermediate formation of acetic acid, and so it might be objected that one ought to be able to identify this acid, as one of the degradation products of sugar by *A. niger*. It must be confessed at once that we have quite failed to do so. Heinze [1903], in a paper previously referred to, states that he obtained acetic acid, in very good yield, along with oxalic acid by the action of *A. niger* on sugar. Unfortunately the lack of experimental details in his paper is so marked—the production of acetic acid being stated as a fact, without any indication as to the method of identification—as to render the statement practically worthless. Obviously the accumulation of acetic acid, and hence the possibility of isolating it, is governed by the relative rates of two reactions:

(*a*) The reaction by which it is produced from sugar.

(*b*) The reaction by which it is decomposed.

Thus if reaction (*a*) proceeds more rapidly than (*b*), acetic acid will accumulate, and one ought to be able to isolate it. If, on the other hand, reaction (*b*) proceeds more quickly than (*a*), then it is obviously impossible, at any stage, to isolate any acetic acid. In other words, in order to explain the non-occurrence of acetic acid, one must be able to show that it is decomposed at least as quickly as is glucose. Fortunately there is perfectly satisfactory evidence to prove that this is the case. W. Pfeffer [1895] in a large series of experiments cultivated *A. niger* in solutions containing glucose, together with one of a number of other substances. He was thus able to compare the relative stability of these substances compared with glucose. His conclusions with regard to acetic acid may be given in (a translation of) his own words. "Although a sufficient amount of glucose is quite capable of more or less protecting glycerol or lactic acid from utilisation, this is not true of acetic acid, which is more freely used by the fungus (*A. niger*) than is glucose itself, when simultaneously present."

It was pointed out in the introduction that, although the non-production of oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent an intermediate stage between sugar and oxalic acid, the production of oxalic acid in quantity from the substance under investigation does not necessarily prove that that substance represents an intermediate stage between sugar and oxalic acid. Nevertheless, such a production of oxalic acid justifies the conclusion that the substance in question probably gives rise, in its decomposition, to a substance which is intermediate between these two compounds. Thus, although succinic, fumaric, malic, and, to a less extent, tartaric acid give rise to good yields of oxalic acid, it is not claimed that each of these substances represents an intermediate stage in the degradation of the sugar molecule. If, however, the scheme suggested is accepted, the production of oxalic acid from these dibasic acids is readily explained. Thus, by a simple reaction, the enol form of oxalacetic acid may be supposed to arise from each acid--and hence oxalic acid:



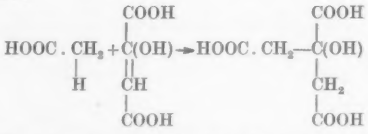
There is experimental evidence, in at least two of these cases, that this change takes place. Wohl and Oesterlin [1901] showed that, by reactions taking place at ordinary temperatures, tartaric acid may be converted into oxalacetic acid. Fenton and Jones [1900] obtained oxalacetic acid from malic acid by oxidation with H_2O_2 and a ferrous salt in the cold.

The principles involved in the suggested scheme also offer an explanation

of the non-production of oxalic acid from the three-carbon acids. The production of a β-ketonic acid, giving rise by hydrolysis to acetic acid, and hence to oxalic acid, is the most essential part of the scheme. If one applies this suggestion to the three-carbon acids, it is seen that no β-ketonic acid, and hence no oxalic acid, can arise from them, since they only contain three carbon atoms. The occasional production of small amounts of oxalic acid from some of them—more particularly from sodium pyruvate in culture media B and C, which were alkaline in reaction—probably can be explained by the fact that secondary changes take place, during sterilisation, which give rise to small amounts of acetic acid.

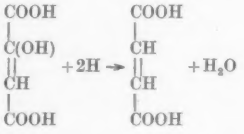
In conclusion, we may be allowed to point out that the scheme suggested for the breakdown of sugar by *A. niger* offers what may be a simple explanation of the production of citric acid by this fungus, and of fumaric acid by *A. fumaricus*.

In stage III of the reactions involved, we have produced one molecule of oxalacetic acid, and one molecule of acetic acid. By the simple union of these two compounds—both of which contain "keten" linkages, which as Collie pointed out readily undergo condensation with one another—a molecule of citric acid would arise:



Citric acid has indeed been synthesised by Lawrence [1897], by condensing oxalacetic ester with bromacetic ester in the presence of zinc. It seems inevitable, in order to explain the production of citric acid from sugar, to presuppose a primary splitting of the sugar molecule, followed by a condensation, because of the presence of a side chain in the citric acid molecule.

The production of fumaric acid might also be easily explained by the reduction of oxalacetic acid:



Summary.

1. *Aspergillus niger* was cultivated on synthetic media containing, as the sole source of carbon, the salts (Na, (NH_4) or Ca) of various organic acids, and the amount of oxalic acid produced was estimated.

2. The following results were obtained:

(*a*) The four-carbon dibasic acids (succinic, fumaric, malic, tartaric) gave good growth, and good yields of oxalic acid.

(*b*) With the four-carbon monobasic acids (butyric, isobutyric, β-hydroxybutyric, hydroxyisobutyric) there was almost no growth, and no production of oxalic acid.

(*c*) The three-carbon acids (lactic, pyruvic, glyceric, malonic, propionic) showed, as a whole, remarkably good growth, but either entire absence, or very small yields, of oxalic acid.

(*d*) Of the two-carbon acids, acetic acid gave good growth, and good yields of oxalic acid. Glycollic and glyoxylic acids gave fairly good growth, but no oxalic acid.

(*e*) The one-carbon acid, formic acid, gave fairly good growth, but no oxalic acid.

3. From a consideration of the results obtained, a theoretical scheme is suggested to represent the breakdown of sugar to oxalic acid, involving the intermediate formation of β.δ-diketo-adipic acid. This undergoes hydrolysis into acetic acid and oxalacetic acid, which on further hydrolysis gives acetic acid and oxalic acid. The acetic acid, which is produced in each case, is then itself oxidised to oxalic acid. It is also suggested that the formation of citric and fumaric acids from sugar, by the Aspergillaceæ, may be referred to the intermediate production of oxalacetic acid.

We take this opportunity of thanking Professor F. Gowland Hopkins most sincerely for his kind encouragement and criticism during the progress of this work.

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XXXIV. ON THE SELF-PURIFICATION OF RIVERS AND STREAMS.

By the late ALBERT EDWIN COOPER; EVELYN ASHLEY COOPER; and JOSEPH ALAN HEWARD.

(*Received September 11th, 1919.*)

It is well known that the purifying action of river-water polluted with sewage is very considerable, as a few miles below the outfall or point of pollution a river may show little or no sign of pollution at all. Purification is effected by sedimentation of the suspended solids, and oxidation of the soluble material. The processes of oxidation give rise to deoxygenation of the river-water, and the extent of deoxygenation depends on the strength of the sewage, the degree of dilution afforded by admixture with the river-water, and the velocity of the river.

If the concentration of oxidisable material be excessive, the river-water will suffer considerable or complete deoxygenation, and a nuisance will result owing to the septic condition caused by the anaerobic decomposition of the organic matter. On the other hand, if there be sufficient dilution, the organic matter can be oxidised and thus destroyed without depriving the river-water of oxygen to any appreciable degree. The suspended matter will also be sedimented in the form of a thin film distributed over a considerable area of river-bed, and no nuisance will thus result through the formation of foul mud-banks.

Recovery from pollution, or self-purification, as it is termed, thus depends on the conditions obtaining with the particular river. Ordinarily towns situated on the same river are sufficiently separated to give time for the river to recover from the effects of the upper pollution before it is subjected to the next. On the other hand, if towns be close together, a nuisance may result, and the river may become unfit to receive a further volume of sewage lower down, until a considerable length of time and dilution from tributaries enable purification to be effected.

As early as 1891 Pettenkofer [see *Roy. Comm.* 1913, p. 65] appreciated the fact that rivers are capable of self-purification and stated that the condition of a river below the point of pollution could be roughly foretold provided the population of the town and the volume of the river were known. He also realised that dissolved oxygen in the river-water played an important part in the process of self-purification.

A certain amount of work has been carried out by the Royal Commission on Sewage Disposal to ascertain the rate of self-purification of polluted river-

waters as measured by the change in the amounts of oxidisable carbonaceous matter and ammonia present, and the production of nitrites and nitrates.

In laboratory experiments it was conclusively shown by Adeney [*Roy. Comm.* 1908] that in mixtures of sewage and river-water the oxidation of the carbonaceous matter precedes that of ammonia. In the case of rivers and streams under natural conditions however the disappearance of the ammonia takes place practically *pari-passu* with the oxidation of the carbonaceous matter [*Roy. Comm.* 1913, p. 63]. This disappearance of ammonia is due to a large extent to absorption by algae and aquatic plants [*Roy. Comm.* 1913, p. 85].

It would also appear that nitrification actually takes place in the polluted river-waters, and this is an additional factor bringing about the disappearance of the ammonia [*Roy. Comm.* 1913, p. 89].

The effect of temperature on the rate of oxidation of organic matter, *i.e.* absorption of dissolved oxygen, was also considered by the Commission [1913, p. 94], especially in relation to the question of standardisation of sewage effluents. Observations were made on the temperatures of different river-waters during the year, and the mean maximum temperature was found to be about 65° F. or 18·3° C. The dissolved oxygen absorption by sewage effluents in five days was found to be much greater at temperatures of 22–24° C. than at 18·3° C., and it was accordingly recommended that in the standardisation of effluents by the dissolved oxygen absorption test a definite temperature should be adopted and that this should be 18·3° C.

Seasonal variation is therefore another factor in the self-purification of rivers. It is clear that owing to the increased rate of oxidation of organic matter due to greater bacterial activity, and the removal of ammonia due to plant development, the process of purification will operate more rapidly during the warmer months. On the other hand, if the river-water be over-charged with sewage, the nuisance may be greater in the summer months than in the winter, as the increased rate of oxidation may lead to deoxygenation of the water.

Although the subject of self-purification of rivers has thus been considerably studied, no work so far seems to have been carried out to ascertain whether the rate and course of purification are influenced by the geological source of the river. Some evidence has already been obtained by the authors [1918] that there is a considerable difference in the rates of oxidation of organic matter in tap-waters and river-waters, the difference being so great that it is necessary to employ the river-water into which the effluent is discharged as the diluting medium in the dissolved oxygen absorption test, in order to obtain results of any value in the standardisation of effluents. It is quite possible that the mineralogical constituents of river-waters may exercise specific influences—either by acceleration or retardation of the oxidation processes.

It may also be expected that the particular types of organic matter derived

from the plant and animal life of rivers and from drainage of land, and the numbers and kind of bacteria and other micro-organisms will have important influences. These however ultimately depend to a large extent on the geological source of the river.

The complex chemical and biological processes going on in river-beds or in the supernatant river-water no doubt have some relation to those going on in soil. There are however differences in the conditions controlling these processes in rivers and on land.

First of all, there is frequently much less humus in a river-bed than in the soil, so that in rivers the chemical and biological processes may be said to be actually going on in a geological bed and uncomplicated by large amounts of decomposed vegetable matter.

Furthermore, in a particular soil there is as a general rule a constancy in the amounts and proportions of the mineralogical constituents. In the case of rivers, on the other hand, except at the spring-head there must be at various times considerable variation, as a river may receive contributions of water from many springs and from drainage off large areas of land of different geological formations, and the relative volume of water from these various sources must depend on climatic conditions and seasonal variations. The conditions obtaining are thus extremely complicated.

Notwithstanding these variations, a river-water may be sufficiently characteristic in chemical composition to distinguish it from other river-waters.

For example, in considering the characters of river-waters in this country, we find that they necessarily depend on the nature of the soils and rocks over which they flow. The rivers of Scotland thus are in general soft, the mountains being composed of granite, gneiss, mica schists, and there is only a small proportion of calcium carbonate. The water from the Cumberland and the Welsh mountains (Silurian rocks) is for the same reason generally soft. In Wales however when the Old Red Sandstone district is reached, marls and limestones are found, and the river-waters are harder. Rivers draining areas in which the Carboniferous Limestone, the Permian Rocks, and New Red Sandstone occur, also yield, as a general rule, somewhat hard water, while river-waters derived from the Lias, Oolitic and Cretaceous Rocks are of necessity hard, because Limestone or Chalk as the case may be are so abundant in these formations.

In draining areas of the various geological strata, rivers carry down an enormous amount of mineral and vegetable matter, which is finally discharged into the sea.

For this reason, river-beds, except possibly near the spring-head, are in general "alluvial" in nature, *i.e.* representative of many geological formations. The mineralogical constitution of a river-bed depends to a large extent on the adjacent geological strata and on those drained by the tributaries of the river, and at different times may be thus liable to considerable variation. Even if the actual mineral constituents in certain cases do not vary much, the physical

and mechanical factors, *i.e.* size and form of particles or texture, would vary and these also would be expected to affect the rate of the chemical and biological processes going on in the river-bed, owing to variations in the degree of aeration.

In the Chalk country, for example, the river-beds will tend to consist of fine-grained calcareous loams or marls, in the Lower Greensand formations these will be replaced by sandy loams, often of coarse texture, sometimes also by peaty loams and fine gravel; again, in the case of a stream from the Lower Greensand formations running over the Atherfield Clay, we should expect to find the clay converted into a sandy clay owing to admixture with the sandy detritus from the Hythe beds. Calcareous, glauconitic and cherty matter might also be found, derived from the Bargate beds, Glauconitic Sandstones, and Chert beds present in the Lower Greensand formation.

There are thus reasons for anticipating that the processes of oxidation of organic and possibly inorganic matter may depend on the quality of the river-water into which it is discharged.

The question is not only of interest to geologists, but is also of importance from the point of view of practical sewage disposal, as, if the oxidation of sewage constituents varies perceptibly in different river-waters, it will be necessary in drawing up new plans for sewage purification to consider the nature of the river into which the effluent is to drain before a standard of purification can be selected.

During the last two years therefore many comparisons of the rates of oxidation in different river-waters of the constituents of sewage or sewage-effluents have been made, and the results of this investigation are given in the present paper. The comparisons have been made by determining the dissolved oxygen absorbed by sewage or sewage effluents when diluted with the various river-waters.

Further experiments supplementary to those described in the previous paper [Cooper, 1918] and comparing the rates of oxidation in the presence of distilled water, various tap-waters and river-waters have also been made.

In addition, some questions of technique bearing on the work and also of importance in the standardisation of effluents are discussed.

The general geological aspect of this work has been studied by the late A. E. C. who unfortunately died in the course of the investigation. In addition, he had proposed to study the mineralogical constitution of the river-beds and to correlate the results with those obtained relating to the processes of oxidation, but this part of the work had only been commenced just before his untimely death and is necessarily not far advanced. His help and enthusiasm in scientific work have been greatly missed. The copious notes on the subject left by him have however been carefully read through, and suitable selections have been incorporated in this paper.

The chemical work has been carried out by E. A. C. and J. A. H. at the Hygienic Laboratory, School of Army Sanitation, Aldershot Command.

1. The estimation of dissolved oxygen in river-waters and diluted sewage effluents.

The method used for the determination of the dissolved oxygen content was that of Winkler, with the Rideal-Stewart modification [*Roy. Comm.* 1913, p. 93].

By this method it was found that aerated distilled water and tap-waters contained 0·93 parts of oxygen per 100,000 at 18·3° C. or 65° F. This is the correct figure according to Roscoe and Lunt's tables [1889].

It was found however that in the case of aerated clean river-waters the oxygen concentration was often somewhat lower, *e.g.* 0·80 to 0·90, while aerated polluted river-waters, and sewage effluents diluted five times with river-water often only contained about 0·80 parts, sometimes, in fact, only 0·70 parts of oxygen per 100,000. As a general rule bad effluents yielded lower results for oxygen concentrations after aeration than good ones, but this was not always the case.

With bad effluents it would not matter whether these low figures were due to experimental error or were realities, as the results would always condemn the effluents. In the case of good or fairly good effluents, the question is of great importance, as is shown by the following examples:

Sewage Effluents.

Initial oxygen concentration after full aeration	Dissolved oxygen absorption in five days at 18·3° C. Parts per 100,000	
	Assuming the oxygen concentrations are correct	Assuming true oxygen concentration is 0·93
1. Effluents 1·5 dilution:		
1. 0·78	0·50	1·15
2. 0·83	0·85	1·25
Canal-water receiving effluent:		
0·91	0·28	0·30
2. Effluents 1·5 dilution:		
1. 0·74	0·20	0·85
2. 0·77	0·60	1·10
Stream-water receiving effluent:		
0·86	0·11	0·18

In the case of good effluents therefore it makes a very considerable difference whether the initial concentration is in reality 0·93 or is the lower figure actually found by experiment.

Both in the routine standardisation of effluents and the carrying out of the investigation on self-purification of rivers it was therefore essential first of all to go into this question of technique.

The low results could be due to at least three factors:

1. The presence of a substance or substances in sewages diminishing the solubility of oxygen in water (in which case the low results would be realities).

2. The presence of oxidisable substances, using up dissolved oxygen or manganese peroxide in the course of the process of estimation.

3. The presence of substances reacting with iodine in the course of the final titration with thiosulphate.

In the latter two cases the actual results obtained for the oxygen concentration are erroneous.

It was thought possible that the sodium chloride present in sewage effluents roughly to the extent of 12 parts chlorine per 100,000 was the cause of the low results. In a sewage effluent diluted 1·5 with stream-water this would yield a chloride concentration of about four parts chlorine per 100,000. Experiments showed however that this concentration of sodium chloride had no effect on the solubility of oxygen in water.

The possible influence of organic matter was then considered. Peptone was dissolved in boiled distilled water just before full aeration and the dissolved oxygen figure of the solution compared with the normal figure at 18·3° C., obtained under the same conditions for distilled water itself. The amount of peptone to be added was judged from the organic nitrogen figure of a fairly bad effluent and amounted to 0·30 g. per litre.

The results obtained were as follows:

Distilled water alone	0·94	0·87	Dissolved oxygen,
Distilled water + 0·3 g. peptone per litre	0·70	0·70	parts per 100,000

It is seen that much lower results were obtained with the peptone solution.

Peptone, and also tank-liquor were next added at various stages of the dissolved oxygen test in order to attempt to ascertain the nature of the interference. The results were as follows:

Dissolved oxygen concentration of boiled distilled water after full aeration at 18·3° C.

1. Distilled water alone (control) ...	0·97	1. Distilled water alone (control) ...	0·92
2. 0·3 g. peptone per litre added before aeration	0·88	2. One-fifth vol. tank-liquor added before aeration	0·85
3. 0·3 g. peptone per litre added immediately after aeration	0·82	3. One-fifth vol. tank-liquor added immediately after aeration ...	0·83
4. 0·3 g. peptone per litre added before precipitation of the manganese peroxide	0·90	4. One-fifth vol. tank-liquor added before precipitation of manganese peroxide	0·75
5. 0·3 g. peptone per litre added just before the peroxide was dissolved by addition of the HCl	0·93	5. One-fifth vol. tank-liquor added just before solution of peroxide in HCl	0·91
6. 0·3 g. peptone per litre added after addition of the acid	0·95	6. One-fifth vol. tank-liquor added after addition of the acid ...	0·84
		7. Tank-liquor alone unaerated contained no dissolved oxygen	

It is seen that the organic matter or other constituents of sewage can affect the results at all stages of the process. The low results do not appear to be due to diminished solubility of oxygen, as the organic matter, etc., exerts its influence after aeration and bottling of the water, and even when added

immediately before the precipitation of the oxygen as peroxide of manganese (probably through decomposition of the latter). It can also affect the titration of the iodine with thiosulphate, probably by reacting quickly with some of the iodine.

The latter point is further illustrated by the following additional results:

	Oxygen concentration of distilled water at 18·3° C.
Distilled water alone	0·90
With addition of tank-liquor just before titration of liberated iodine with thiosulphate	0·84

Further experiment also showed that, if a diluted sewage effluent were aerated and allowed to stand for a short time before estimation of the dissolved oxygen, there was also a marked fall in oxygen concentration:

	Immediately after aeration	Standing 40 mins. after aeration
Oxygen concentration	0·86	0·66
„ „	0·88	0·79

Such rapid loss of oxygen must probably be due to chemical oxidation of certain substances, *e.g.* ferrous sulphide and organic matter, and points to this factor as one cause of the low results obtained in estimating the dissolved oxygen content of sewage effluents.

The presence of suspended matter in sewage effluents on the other hand seemed to have only a slight effect on the dissolved oxygen figure:

	Parts per 100,000	
Unfiltered	0·84	0·85
Filtered	0·87	0·84

From these results it would appear that the low results obtained in estimating the dissolved oxygen content of aerated sewage effluents are due to:

1. Rapid oxidation of certain constituents during the estimation.
2. Decomposition of a portion of the manganese peroxide by organic matter.
3. Loss of iodine in the final thiosulphate titration due to reaction with constituents of sewage.

Attempts were next made to destroy the constituents of effluents causing the interference by (1) doubling the amount, and (2) doubling the time of action of the permanganate used in the Rideal-Stewart modification of Winkler's dissolved oxygen test. The permanganate is primarily used to destroy nitrites, which also interfere with the test, but it was thought that it might also be serviceable in destroying the other interfering substances if sufficient concentration or time were allowed.

Satisfactory results were sometimes but not always obtained when the amount of permanganate was doubled:

Oxygen concentration. Parts per 100,000.

Usual amount of permanganate	Double amount
0·87	0·98
0·88	0·94
0·81	0·81
0·71	0·77
0·70	0·81
0·82	0·82
0·88	0·90
0·83	0·91
0·86	0·83
0·88	0·93
0·88	0·935
0·85	0·90

Doubling the time, *i.e.* allowing 40 mins. instead of 20 mins., also did not give good results:

Usual time	Double time
0·83	0·86
0·85	0·84
0·80	0·91
0·74	0·78

There thus seemed to be no simple method of preventing the low results obtained in estimating the oxygen content of sewage effluents.

Experiments were therefore carried out to ascertain if the error still existed after incubation of the diluted effluent at 18·3° C. as carried out in the Rideal-Stewart or dissolved oxygen absorption test. If the interfering substances were largely oxidised or otherwise destroyed during the incubation, there would obviously be a large error in the initial oxygen estimation, but little or none in the final estimation after five days. In this case, the results for the dissolved oxygen figures of the effluents would be low and a considerable error thus introduced in routine work. On the other hand, if the interfering substances were not appreciably destroyed, the error in estimation would be practically the same at the end of five days as at the commencement of the test, and would thus be neutralised, and sufficiently accurate results would be obtained for ordinary purposes.

Experiments were therefore carried out to throw some light on this matter. Aerated diluted effluents were incubated in closed bottles at 18·3° C. and after five days or other suitable period the contents of the bottles were again *reaerated* strictly at the same temperature and the oxygen concentrations estimated and compared with the initial result.

It is seen that as a general rule there was very little difference in the figures, and, thus, although low results are often obtained in the estimation of oxygen in sewage effluents, the error is neutralised and does not interfere with the utility of the dissolved oxygen test.

Initial oxygen concentration at 18·3° C.	Oxygen concentration after incubation and aeration at 18·3° C.
Parts per 100,000	
0·91	0·90 (5 days)
0·90	0·90 (5 „)
0·87	0·88 (5 „)
0·86	0·85 (5 „)
0·83	0·82 (24 hours)
0·80	0·80 (3 days)
0·80	0·73 (13 „)
0·88	0·82 (24 hours)
0·88	0·86 (7 days)
0·88	0·90 (5 weeks)
0·87	0·86 (5 days)
0·73	0·73 (4 weeks)
0·85	0·94 (4 „)
0·84	0·87 (5 days)
0·80	0·83 (2 „)

2. The effect of temperature on the dissolved oxygen absorption figures of effluents.

Reference has already been made to the experimental work of the Sewage Commission, showing that the oxidation processes were considerably accelerated by rise in temperature from 18·3° to 22° or 24° C., on account of which it was considered essential to carry out the dissolved oxygen absorption test rigidly at 18·3°, the average maximum temperature of rivers in this country.

Some further experiments have been made on the question of the influence of temperature, and are summarised below:

Dissolved oxygen absorption figures for effluents.
Parts per 100,000, five days.

10° C.	18·3° C.	22° C.	37° C.
1·10	1·75	—	—
1·20	2·45	—	—
0·30	0·60	—	—
—	0·65	2·10	—
—	9·80	9·70	9·6
—	11·30	11·10	11·00

(In all these experiments the initial aerations were carried out strictly at the particular temperatures selected.)

It is seen that in one case out of three a very much higher result was obtained at 22° than at 18·3°, while in the two other cases at temperatures of 18·3°, 22°, 37°, no differences were observed. At 10° however the oxidation processes went on very much more slowly than at 18·3°, and the results emphasise the necessity of carrying out the test under strictly standardised conditions. For routine work the standard temperature of 18·3° C. recommended by the Commission has been used.

The figures tabulated above are also of interest from the point of view of the general question of river purification, as they tend to show that sewage will have a considerably less polluting effect in the winter months than during the summer, while it will not necessarily cause a greater pollution in rivers in tropical countries than in temperate regions.

3. The effect of time on the dissolved oxygen absorption test.

The period of five days was recommended by the Commission for this test [*Roy. Comm.* 1913, p. 78], as it was suspected that unreliable results were obtained if shorter periods were employed, owing to the possibility of a bacterial lag, and a greater influence due to the presence of inhibitory substances in the sewage. The value of the test, however, would be considerably enhanced, if a shorter period than five days would be sufficient. It was therefore considered advisable to carry out more experiments on this point, and the results are tabulated below:

Dissolved oxygen absorbed at 18·3° C.

	48 hours	5 days	Ratio
1. Effluent	0·14	0·30	1 : 2
Stream above effluent	0·007	0·014	1 : 2
Stream below effluent	0·02	0·11	1 : 5
2. Effluent	0·70	1·00	1 : 1·4
Stream above ...	0·04	0·10	1 : 2·5
3. Primary effluent ...	1·70	3·00	1 : 1·7
4. Effluent	0·12	0·95	1 : 8
Stream above ...	0·12	0·13	1 : 1
5. Effluent	2·35	All O absorbed	1 : ?
6. Effluents 1	0·10	0·80	1 : 8
„ 2	0·00	1·45	1 : ∞
Stream above ...	0·16	0·29	1 : 1·8
7. Effluents 1	0·00	0·70	1 : ∞
„ 2	0·18	>3·45	1 : >19
„ 3	0·16	0·75	1 : 4·7
„ 4	0·46	>3·40	1 : >7·4
River above	0·11	0·23	1 : 2
8. Effluents	0·80	0·65	1 : 0·8
„	1·20	1·55	1 : 1·3
Stream	0·08	0·26	1 : 3

The Commission found that the five days' test gave a very useful idea of the polluting effect of an effluent [*Roy. Comm.* 1913, p. 63], and it was thought that if a fairly definite ratio between the two and five days' tests could be obtained, it would be possible to use the two days' test as a standard. The above results however show that this is not the case, as even very bad effluents may give little or no indication of oxygen absorption in two days.

Further experiments using a stronger concentration of effluent, *i.e.* 1 : 1 dilution with river-water, were next carried out. The results are as follows:

	48 hours	5 days	Ratio
1. Effluent	0·15	1·40	1 : 9
2. „	0·56	0·90	1 : 1·6
Stream above ...	0·09	0·15	1 : 1·7
3. Effluent 1	0·52	0·65	1 : 1·2
„ 2	0·96	1·55	1 : 1·6
Stream	0·08	0·26	1 : 3·2

Even with the increased concentration it is seen there is considerable variation in the ratio, and at the present time there is no alternative but to employ the longer period of five days.

It was thought that it might be possible to accelerate the processes by means of a catalyst, and in this connection the effect of manganese salts has been studied. Small amounts of manganese chloride were added to diluted sewage effluents, the dissolved oxygen contents estimated at the end of two days, and the results compared:

	Dissolved oxygen absorbed in 2 days. Parts per 100,000
Effluent without $MnCl_2$	0·65
„ plus 0·1 % $MnCl_2$	0·02
„ plus 0·025 % $MnCl_2$	0·03

The addition of the manganese salt thus considerably inhibited the absorption of the dissolved oxygen. Further experiments with other catalytic substances are desirable.

4. The influence of dilution.

The Commission [*Roy. Comm.* 1913, p. 80] carried out some experiments bearing on the question of the effect of dilution on the rate of dissolved oxygen absorption by sewage effluents, and found that dilution up to 1 in 3 with tap-water had very little effect. It was thought desirable to carry out some more experiments, and the results are illustrated by the following figures:

Dilution	Dissolved oxygen absorbed by effluent. Parts per 100,000	
1 in 2	0·52	0·96
1 in 5	0·80	1·20

It is seen that the oxygen absorption is slightly increased by increasing the dilution $2\frac{1}{2}$ times. Increasing the dilution from 1 in 5 to 1 in 20 had practically no influence at all, and dilution would thus seem to be not such an important factor as would be expected.

5. The possible influence of inhibition factors.

The possible influence of certain constituents, *e.g.* lime, in sewage in inhibiting the absorption of dissolved oxygen was realised by the Commission [*Roy. Comm.* 1913, p. 84]. It was considered quite likely that the dissolved

oxygen test might for this reason under certain conditions give false and low results, especially if the two days' test was used instead of the five days' test. The possibility of ammonia acting as inhibitory agent does not however appear to be discussed. It has been noticed frequently in routine work that an effluent containing as much as 10 parts per 100,000 of ammoniacal nitrogen might yield a dissolved oxygen absorption figure of only about 1·0 and would thus be classified as a satisfactory effluent. Experiments have been therefore carried out to ascertain whether ammonia in such concentrations has any inhibitory action. Ammonium carbonate was used for this purpose:

Diluting medium 1 : 5	Dissolved oxygen absorbed by an effluent in 5 days
Distilled water	13·0
„ containing amm. carb. equivalent to 2·5 parts amm. N per 100,000	13·4
„ „ „ „ „ 5·0 „ „ „	13·4
„ „ „ „ „ 10·0 „ „ „	12·6
„ „ „ „ „ 20·0 „ „ „	12·2
„ „ „ „ „ 45·0 „ „ „	11·2

It is seen that ammonia in concentrations such as occur in sewage effluents had no perceptible influence on the rate of oxygen absorption. Much larger concentrations in fact had only a small effect.

6. A COMPARISON OF THE CAPACITIES FOR SELF-PURIFICATION OF DIFFERENT RIVER-WATERS.

This comparison has been made by measuring the rate of oxidation of the constituents of sewage effluents or unpolluted river-muds in the presence of various river-waters. The sewage effluents were diluted with the river-waters, and the mixtures incubated at 18·3° C. usually for five days as in the dissolved oxygen absorption test. The amount of oxygen absorbed by the diluted sewage and also by the unpolluted river-water itself being thus determined by Winkler's process, the dissolved oxygen absorption due to the sewage or effluent could be ascertained by difference[1]. The experiments with the muds were similarly carried out.

The geological source and nature of the river-bed has been investigated in the case of each river, and the conditions have been correlated with the results of the oxidation experiments. The results are set forth in the following table. Unless otherwise stated, the temperature of the incubation was 18·3°, dilution of effluent 1 : 5, and period of incubation five days.

[1] In the case of tank-effluents, the amounts used were so small and the dilution thus was so great that the actual absorption of dissolved oxygen by the river-water itself was not required, because the small amount of sewage did not affect the estimation of oxygen, and the absorption of oxygen by the tank-effluent could be determined by taking the difference between the oxygen concentrations of the river-water, and river-water + effluent at the end of the period of incubation. This was also the case in the experiments with muds, as before titration the supernatant water was syphoned off from the layer of mud into another stoppered bottle.

Comparison of rate of oxidation in river-waters.

Material used	River (1) Dissolved oxygen absorbed, parts per 100,000, by: Effluent when diluted with this water	River (1) Dissolved oxygen absorbed, parts per 100,000, by: River-water alone	River (1) Character of river-bed at point of sampling	River (2) Dissolved oxygen absorbed, parts per 100,000, by: Effluent when diluted with this water	River (2) Dissolved oxygen absorbed, parts per 100,000, by: River-water alone	River (2) Character of river-bed at point of sampling
(*a*) 1. From Gravel and Upper Bagshot Sands.						
2. From same beds but after river has subsequently run over, first Bracklesham beds, Lower Bagshot and finally London Clay for a considerable distance.						
Land-effluent	1·35	0·19	Gravel and sand	1·70	0·02	Clay
,,	0·55	0·19	,,	1·85	0·02	,,
,,	0·00	0·19	,,	0·95	0·02	,,
,,	2·61	0·00	,,	2·19	0·11	,,
,,	2·68	0·00	,,	2·05	0·11	,,
,,	0·94	0·00	,,	0·67	0·11	,,
,,	1·04	0·00	,,	0·68	0·11	,,
,,	1·05	0·09	,,	0·95	0·19	,,
,,	0·60	0·09	,,	0·55	0·19	,,
,,	1·05	0·18	,,	1·60	0·23	,,
,,	1·20	0·08	,,	1·10	0·53	,,
,,	0·65	0·00	,,	1·15	0·22	,,
,,	0·80	0·04	,,	0·65	0·13	,,
,,	1·60	0·04	,,	0·45	0·13	,,
Tank-effluent (dilution 1 in 50) 2 days	13·75	—	,,	{36·25 / 30·25}	—	,,
,, 1 ,,	6·50	—	,,	11·10	—	,,
,, 2 ,,	13·75	—	,,	14·75	—	,,
,, 1 ,,	17·00	—	,,	16·00	—	,,
,, 1 ,,	4·00	—	,,	6·00	—	,,
,, 3 ,,	9·00	—	,,	13·40	—	,,
,, 5 ,,	10·50	—	,,	15·20	—	,,
,, 2 ,,	23·50	—	,,	21·70	—	,,
,, 3 ,,	28·50	—	,,	14·50	—	,,
,, 4 ,,	34·50	—	,,	24·50	—	,,
River-mud (dilution 1 in 100)	63·3	—	,,	70·00	—	,,
,,	65·0	—	,,	58·30	—	,,
(*b*) 1. From Gravel and Upper Bagshot Sands.						
2. From same beds but mixed with river-water from Bracklesham beds.						
Land-effluent	0·35	0·13	Gravel and sand	1·05	0·24	Sand and clay
,,	0·65	0·15	,,	0·00	0·27	,,
Tank-effluent (dilution 1 in 50) 2 days	22·25	—	,,	10·75	—	Green (Glauconitic) clay
,, 2 ,,	26·00	—	,,	17·15	—	,,
,, 2 ,,	10·75	—	,,	11·62	—	,,
,, 4 ,,	14·25	—	,,	17·00	—	,,
Glauconitic mud from river in Bracklesham beds (dilution 1 in 100)	63·30	—	,,	55·80	—	,,
,,	65·00	—	,,	53·70	—	,,
Land-effluent	0·65	0·09	,,	0·00	0·11	Clay and sand
,,	0·50	0·06	,,	0·60	0·17	,,
(*c*) 1. From Gravel and Upper Bagshot Sands.						
2. From Lower Bagshot, subsequently running over London Clay.						
Effluent	1·95	0·11	Gravel and sand	3·35	0·17	Clay
,,	0·18	0·03	,,	1·35	0·22	,,

Comparison of rate of oxidation in river-waters.

Material used		Dissolved oxygen absorbed, parts per 100,000, by: Effluent diluted with this water	River-water alone	Character of river-bed at point of sampling	Dissolved oxygen absorbed, parts per 100,000, by: Effluent diluted with this water	River-water alone	Character of river-bed at point of sampling
(*d*)		1. From Folkestone beds.			2. From Hythe beds.		
Land-effluent		1·35	0·09	Sand	0·85	0·21	Sand
,,		1·35	0·13	,,	0·95	0·18	,,
,,		>3·75	0·12	,,	1·90	0·30	,,
,,		1·70	0·12	,,	1·10	0·30	,,
,,		0·50	0·08	,,	0·30	0·20	,,
,,		0·65	0·28	,,	1·15	0·29	,,
,,		0·65	0·28	,,	0·55	0·26	,,
,,		0·65	0·38	,,	1·40	0·33	,,
,,		0·90	0·38	,,	1·40	0·33	,,
,,		0·90	0·16	,,	1·85	0·23	,,
Tank-effluent (dilution 1 in 50)	1 day	38·5	—	,,	41·25 42·5	—	,,
,,	1 ,,	40·0	—	,,	45·5	—	,,
,,	2 ,,	14·0	—	,,	30·50	—	,,
,,	2 ,,	15·0	—	,,	30·50	—	,,
,,	1 ,,	28·75	—	,,	28·50	—	,,
,,	2 ,,	41·30 42·10	—	,,	44·1	—	,,
,,	1 ,,	27·25	—	,,	11·65	—	,,
,,	2 ,,	38·00	—	,,	18·00	—	,,
(*e*)		1. From Folkestone beds.			2. From Folkestone beds, subsequently running over Sandgate beds.		
Land-effluent		1·70	0·12	Sand	2·66	0·11	Sand
,,		1·55	0·12	,,	1·90	0·11	,,
,,		1·15	0·09	,,	0·90	0·15	,,
Tank-effluent (dilution 1 in 50)	1 day	9·50	—	,,	12·50	—	,,
,,	2 ,,	11·50	—	,,	23·50	—	,,
,,	1 ,,	9·54	—	,,	8·92	—	,,
,,	3 ,,	16·40	—	,,	17·75	—	,,
,,	5 ,,	18·30	—	,,	21·60	—	,,
,,	2 ,,	11·75	—	,,	11·90	—	,,
,,	3 ,,	16·50	—	,,	11·75	—	,,
(*f*)		1. Gravel and Upper Bagshot Sands.			2. Lower Greensand.		
Land-effluent		1·00	—	Gravel and sand	0·45	—	Sand
,,		1·90	—	,,	1·20 (Folkestone beds)	—	,,
Stream-mud (from Hythe bed stream) (dilution 1 in 60)		23·00	—	,,	31·25	—	,,
,,		34·00	—	,,	33·00 (Hythe beds)	—	,,
Land-effluent		1·80	0·03	,,	1·95 (Hythe beds)	0·28	,,
,,		2·25	0·08	,,	2·40 (Folkestone beds)	0·21	,,
,,		0·50	0·13	,,	0·70 (Hythe beds)	0·17	,,

Comparison of rate of oxidation in river-waters.

(*g*) From Gravel, Sand, and Clay, and from the Chalk.

Material used		Dissolved oxygen absorbed, parts per 100,000, by: Effluent diluted with the river-water	River-water alone	Character of river-bed at point of sampling	Dissolved oxygen absorbed, parts per 100,000, by: Effluent diluted with the river-water	River-water alone	Character of river-bed at point of sampling
		River-water from Lower Bagshot Clay, subsequently running over London Clay.			River-water from Chalk subsequently running over Woolwich and Reading beds.		
Land-effluent		1·15	0·23	Clay	1·70	0·06	Calcareous gravel
,,		0·90	0·23	,,	0·80	0·06	,,
		River-water from Lower Chalk.			River-water from Folkestone beds subsequently running over Sandgate beds.		
,,		2·60	0·09	Marly loam and calcareous gravel	1·45	0·19	Sand
		River-water from Gravel and Upper Bagshot Sands.			River-water from Chalk subsequently running over Woolwich and Reading beds.		
Tank-effluent (dilution 1 in 50)	1 day	24·9	—	Gravel and sand	32·0	—	Calcareous gravel
,,	2 ,,	34·4	—	,,	41·3	—	,,
,,	3 ,,	40·0	—	,,	45·7	—	,,
		River-water from Gravel and Upper Bagshot Sands, subsequently running over Bracklesham beds, Lower Bagshots and London Clay.			River-water from Chalk subsequently running through Woolwich and Reading beds.		
Tank-effluent (dilution 1 in 50)	1 day	11·40	—	Clay	6·00	—	Calcareous gravel
,,	2 ,,	9·50	—	,,	8·80	—	,,
,,	3 ,,	10·60	—	,,	3·20	—	,,
		River-water from Lower Bagshot subsequently running over London Clay.			River-water from Chalk subsequently running over Woolwich and Reading beds (3 different rivers).		
Filter-effluent		1·35	0·22	Clay	1·15	0·05	Calcareous gravel
,,		River-water from Gravel and Upper Bagshot Sands.			0·75	0·02	,,
,,		0·18	0·03	Gravel and sand	1·08	0·11	,,
		River-water from Gravel and Upper Bagshot Sands.			River-water from Lower Chalk, Upper Greensand and Gault subsequently running over Folkestone beds.		
Tank-effluent (dilution 1 in 50)	1 day	9·50	—	Gravel and sand	10·25	—	Clay
,,	2 ,,	11·25	—	,,	19·87	—	,,
,,	3 ,,	16·75	—	,,	22·70	—	,,
		River-water from Folkestone beds subsequently running over Sandgate beds.			River-water from Lower Chalk, Upper Greensand and Gault subsequently running over Folkestone beds.		
Filter-effluent		11·40	0·16	Sand	9·85	0·11	Clay
		River-water from Sandgate beds, Lower Greensand.			River-water from Lower Chalk and Upper Greensand.		
Effluent		0·80	0·04	Sand	0·45	0·00	—

A survey of the tabulated results shows that very considerable differences exist in the rate of oxidation of the constituents of sewage effluents in different river-waters of different geological source. There is however a marked inconsistency in the results, so that it is not yet possible to draw any definite conclusions as to the influence of geological source on the oxidation processes in river-waters. This inconsistency may be expected, as the conditions, *i.e.* the chemical composition and bacterial population of sewage are subject to very great variation, and thus in each experiment new factors may be introduced.

The results so far obtained may be summarised thus:

Although in the case of nearly all the river-waters used for comparisons, close agreement in the rates of oxidation was sometimes observed, yet marked differences were frequently found.

Oxidation proceeded more slowly or more quickly in:

1. River-water from the Gravel and Upper Bagshot Sands than in river-water derived from the same source but subsequently running over successively the Bracklesham beds, Lower Bagshot Sands, and London Clay.

2. River-water from the Gravel and Upper Bagshot Sands than in river-water from the same beds, but mixed with river-water from the Bracklesham beds.

3. River-water from the Folkestone beds than in river-water from the Hythe beds.

4. River-water from the Folkestone beds than in river-water derived from the same beds but subsequently running over the Sandgate beds.

5. River-water from the Gravel and Upper Bagshot Sands than in river-water from the Lower Greensand strata.

6. River-water from Gravel, Sand, and Clay than in river-water from the Chalk.

7. Oxidation proceeded more slowly in river-water from the Gravel and Upper Bagshot Sands than in river-water from the Lower Bagshot Sands and London Clay.

A considerable amount of work has yet to be done in order to interpret these variable results. They indicate however the importance of always strictly using the particular river-water into which an effluent is discharged for dilution of the effluent in Winkler's dissolved oxygen absorption test. This rule should always be adhered to, unless it can be conclusively shown by a long series of experiments that any other river-water, which happens to be more convenient for use, gives results in close agreement.

No work seems to have been carried out previously on the deoxygenating action of the muds of unpolluted rivers. The experiments described in this paper show that such river muds can absorb from 12 to 70 parts per 100,000 of oxygen from river-waters in five days at 18·3° C.

Further observations are given below:

Character of mud	Supernatant water used	Dissolved oxygen absorbed. Parts per 100,000
From clean bed of stream formed by spring-water from Hythe beds thrown out by Atherfield Clay	Stream water	6·00
From stream in Folkestone beds	Distilled water	16·00
From river in Sandgate beds	,,	40·00
From river in London Clay	River-water	44·00

It is seen that unpolluted river-muds absorb very considerable amounts of dissolved oxygen, and their potential deoxygenating effect is in every case much greater than that of a sewage effluent of fair quality (2 parts per 100,000). As a general rule however muds of unpolluted rivers will have actually less deoxygenating effect than sewage effluents, because the volume of the superficial deoxygenating layer of mud relative to that of the river is small.

7. Comparison of the dissolved oxygen absorption of sewage effluents when diluted with various river- and tap-waters.

In a previous communication [Cooper, 1918] it was shown that the dissolved oxygen absorption figures of effluents were considerably lower when dilution was made with a hard tap-water from the Chalk than with distilled water or river-water taken from the river receiving the effluent. It was thought desirable to obtain more data bearing on this question and comparisons of the rates of oxidation in the presence of: 1. River-water above the outfalls. 2. Tap-water at laboratory. 3. Local tap-water, *i.e.* water-supply running into sewerage.

Some results on these lines have already been given in Section 6 of this paper, as the river-water from the Gravel and Upper Bagshot Sands happened to be derived from the same springs as yielded the laboratory tap-water and the results thus serve as comparisons of dissolved oxygen absorption in the presence of tap-water (Gravel and Upper Bagshots) and the river-waters.

It is seen that the oxygen absorption often varied considerably and quite different results were obtained according as tap- or river-water was used for dilution. Further comparisons are made in the table on p. 362.

It is seen that lower results were frequently obtained with the various hard calcareous waters, thus confirming the previous findings. This however was not invariably the case.

The fact that hard waters do not always inhibit the oxidation processes is further brought out by the following experiments with river-muds and effluents:

	Supernatant water	Dissolved oxygen absorption mud or effluent. Parts per 100,000	
1.	Distilled water	16	
	Hard water from Chalk	12	
	,, ,, ...	20	
2.	Distilled water	40	
	,, plus solid calcium carbonate ...	39	
	Hard water from Chalk	30	
		a.	*b.*
3	Effluent diluted with distilled water	1·20	0·90
	,, ,, ,, ,, plus Oolite	1·05	0·95
	,, ,, ,, ,, plus Barytes	0·90	0·75

Dissolved oxygen absorption. Parts per 100,000 *of effluent.*

	Diluted with river-water receiving effluent	Diluted with laboratory tap-water	Diluted with local tap-water, *i.e.* water-supply running into sewerage
1.	36·25	28·0	—
	30·25 (London Clay)	23·0 (Hard water from Chalk beneath Tertiary formations)	—
2.	0·55 (London Clay)	—	1·90 (Hard water from Chalk beneath Tertiary formations)
	0·00 (Gravel and Upper Bagshot Sands)	—	7·75 (Hard water from Chalk beneath Tertiary formations)
	23·0 (Gravel and Upper Bagshot Sands)	23·0 (Hard water from Chalk beneath Tertiary formations)	—
	0·00 (Bracklesham beds)	0·65 (Gravel and Upper Bagshots)	—
	0·90 (Sandgate beds)	—	1·15 (Hythe beds)
	0·30 (Gravel and Upper Bagshot and Bracklesham beds)	—	0·35 (Chalk beneath Tertiaries)
	20·7	19·5	—
	23·75 (Gravel and Upper Bagshots)	24·35 (Gravel and Upper Bagshots)	—
	3·35 (London Clay)	1·95 (Gravel and Upper Bagshots)	2·80 (Chalk beneath Tertiary formations)
	,,	,,	1·30 (Tap-water from another local supply, water from Chalk at surface)
	0·65 (Bracklesham beds)	—	0·45 (Chalk beneath Tertiaries)
	1·90 (London Clay)	—	1·05 (Chalk beneath Tertiaries and river gravel)
	1·35 (London Clay)	0·18 (Gravel and Upper Bagshots)	0·90 (Chalk beneath Tertiaries and river gravel)
	,,	,,	1·05 (Upper Chalk, another local supply)

8. Comparison of dissolved oxygen absorption of sewage effluents diluted with river-water and distilled water.

In the previous paper [Cooper, 1918] some evidence was offered showing that the dissolved oxygen absorption figures of sewage effluents were higher when distilled water was used for dilution than when river-water was em-

ployed. Since then many other experiments have been made and the results are recorded below:

Dissolved oxygen absorption. Parts per 100,000 *of effluent.*

1. Diluted with river-water receiving effluent		2. Diluted with distilled water
0·0	(Gravel and Upper Bagshot Sands)	7·65
23·00	„ „ „	15·50
1·80	„ „ „	1·50
0·65	„ „ „	1·25
1·10	„ „ „	0·95
2·25	„ „ „	2·40
0·18	„ „ „	1·30
0·50	„ „ „	0·85
1·15	(London Clay)	1·25
1·35	„	1·30
2·05	„	2·00
2·35	„	2·75
0·80	(Sandgate beds—Lower Greensand)	0·45
1·25	„ „ „	1·25
1·95	(Hythe beds—Lower Greensand) ...	1·50
0·70	„ „ „ ...	0·85
1·40	(Folkestone beds—Lower Greensand)	1·40
2·40	„ „ „	2·40
0·45	(Lower Chalk and Upper Greensand)	0·45
1·15	(Chalk and Tertiaries)	1·30
0·75	„ „	1·30
1·08	„ „	1·30

The results show that there is sometimes close agreement in the two sets of figures, and in this case for practical purposes it makes no difference whether distilled or river-water is used. In many cases however there is a considerable discrepancy and oxidation may go on either more slowly or more quickly in river-water than in distilled water. In the case of water from the Gravel and Upper Bagshot Sands the inhibitory effect was often very marked, and was greater in fact than shown in the earlier results [Cooper, 1918]. Oxidation also proceeded more slowly in river-waters from the Chalk than in distilled water.

River-water from the London Clay on the other hand as a rule had no perceptible inhibitory or accelerating effect on the oxidation processes. In one experiment however there was an appreciable inhibition. In the case of river-waters from the Lower Greensand (Sandgate, Folkestone, and Hythe beds) the results were variable. The earlier experiments [Cooper, 1918] pointed to a considerable inhibitory action, but in some of the further experiments described above oxidation proceeded either more rapidly in the river-waters or at the same rate approximately as in distilled water.

9. Comparison of dissolved oxygen absorption of sewage effluents when diluted with various tap-waters.

In the previous communication it was shown that the constituents of sewage effluents oxidised about 5 to 20 times more rapidly in distilled water than in a hard tap-water, and about 25 per cent. faster in distilled than in tap-water from the Gravel and Upper Bagshot Sands.

In the case of a highly ferruginous tap-water from the Folkestone beds there was an initial inhibition which however was no longer noticed when the iron was removed by oxidation and filtration. In the usual five days' dissolved oxygen test, on the other hand, oxidation proceeded at the same rate in the tap-water (filtered or unfiltered) as in distilled water.

Some further observations on the relative rate of oxidation in distilled water, hard tap-waters, and tap-water from the Gravel and Upper Bagshot Sands are given below:

Dissolved oxygen absorbed in five days at 18·3° C.

Material used	Dilution with		
	Distilled water	Hard water	Soft water from Gravel and Upper Bagshot
Primary filter-effluent	1·30	0·90	0·18
" "	1·30	1·05	0·18
" "	—	2·80	1·95
" "	—	1·30	1·95
Tank-liquor	—	7·75	0·00
"	—	23·00	23·00
Land-effluent	—	0·35	0·30

Additional figures affording comparisons of oxidations in distilled water and tap-water from Gravel and Upper Bagshot Sands are given in Section 8, the river-water from these geological formations being derived from the same springs as supplied the tap-water.

A survey of all the results shows that there is very great variation in the relative rate of oxidation of sewage constituents in distilled water and in the tap-waters (soft and hard). It is seen that both tap-waters frequently exercise a very considerable inhibitory effect on the rate of oxidation, and that the hard water may have either a greater or smaller inhibitory action than the soft water. In some cases however there is a fairly close agreement. The results point to the conclusion that the oxidation processes in the artificial methods of sewage purification must be considerably affected by the nature of the water-supply which mixes with the sewage, and also by that of surface-water gaining access to the drainage system. Owing to the great variations it is not possible to draw any helpful conclusions from the results of laboratory work in reference to this question, but it may be pointed out that the possibility of inhibitory action should always be borne in mind in framing new schemes for sewage purification, and arrangements should always be made to render it easily possible to extend the area of filtration, should difficulties be encountered.

10. The oxygen absorbed from permanganate or "Tidy Test."

The test is used extensively in the chemical examination of water supplies. It is also employed to a certain extent in the standardisation of sewage effluents, although the Sewage Commission [*Roy. Comm.* 1913, p. 74] has reported that it gives a less correct measure of the polluting effect of an effluent than the dissolved oxygen test. Some observations made in the course of water analysis at first suggested that geological factors influenced the permanganate test just as they had been found to affect the Winkler's test. Thus, the loss in strength of the permanganate solution on incubation was occasionally found to be greater in the presence of distilled water than in presence of pure tap-waters (calcareous or soft water supplies).

10 *cc. N*/80 *potassium permanganate.* 10 *cc.* 25 % *sulphuric acid.* 100 *cc. water. Four hours at* 37° C.[1]

Immediate titration	Titration with thiosulphate after 4 hrs incubation with distilled water	Titration after 4 hrs incubation with pure tap-water
12·4 cc.	11·2 cc.	11·8 cc. (hard water)
15·0	13·2	13·8 „
15·4	15·0	15·3 (soft water)

It would seem that certain constituents of the tap-water inhibit either the natural decomposition of the permanganate or the oxidation of some impurity present in it [Cooper and Heward, 1919]. Experiments were therefore next carried out to ascertain if the rate of oxidation of the constituents of sewage by permanganate were affected by dilution with different tap-waters. For this purpose the Tidy Test was carried out in the usual way.

10 *cc. Tank Liquor* (*Sedimented Sewage*). 10 *cc. N*/80 *permanganate.* 10 *cc.* 25 % *sulphuric acid.* 90 *cc. water. Four hours at* 37° C.

Oxygen absorbed by Tank Liquor from Permanganate. Parts per 100,000.

Dilution with distilled water	Dilution with hard calcareous water	Dilution with soft peaty water
3·10	3·00	3·50
2·00	2·10	—
7·25	7·40	—
8·00	—	8·20
3·70	—	2·33

The results as a whole show that the permanganate test unlike the dissolved oxygen test is not appreciably affected by the presence of a hard or soft water. It follows from this that the permanganate test gives a more correct measure of the actual amount of oxidisable matter present, and is thus of special value in water analysis. The dissolved oxygen test on the other hand determines the amount of oxidation that can go on under certain defined conditions, and thus measures, not the proportion of oxidisable matter present, but its actual deoxygenating or polluting effect on the diluting water. This test will thus be of special value in the standardisation of sewage effluents.

[1] 37° C. was used as standard temperature as it was more convenient and as there was practically no difference in the results obtained at 27° and 37°.

SUMMARY.

1. Fully aerated sewage effluents and polluted river-waters may contain considerably less dissolved oxygen than distilled water under the same experimental conditions. The low results appear to be due to various factors, and cannot be prevented by any method so far employed. As, however, the oxygen content is still the same after incubation for five days and subsequent re-aeration, there seems to be no appreciable error involved in Winkler's dissolved oxygen absorption test (Rideal-Stewart modification).

2. The oxidation of the constituents of sewage proceeds much more slowly at 10° C. than at the standard temperature of 18·3° C. usually employed.

At temperatures of 22° C. and 37° C. oxidation may proceed either at the same rate as at 18·3° C. or sometimes faster. The results show the necessity of adhering strictly to a definite temperature in the dissolved oxygen absorption test. The temperature of 18·3° C. was recommended by the Sewage Commission, this being the maximum temperature of river-waters in this country.

3. Attempts to shorten the five days' dissolved oxygen absorption test to two days have not been successful, as owing to an initial lag most variable ratios between the two and five days' tests are obtained. A bad effluent in fact may give very little indication of oxygen absorption in two days.

4. Dilution appears to have comparatively little influence on the rate of oxidation of the constituents of sewage.

5. Ammonia up to a concentration of 45 parts per 100,000 has also only a slight inhibitory effect on the rate of oxidation.

6. Oxidation of sewage constituents may proceed at very considerably different rates in river-waters of different geological source. The results are however very variable, and it is not yet possible to draw any definite conclusions as to the influence of geological factors upon the oxidation processes.

While in the case of nearly all river-waters employed, close agreement in the results is sometimes observed, oxidation may be slower or quicker:

(i) In river-water from the Gravel and Upper Bagshot Sands than in river-water from the London Clay, Bracklesham beds, and Lower Greensand formation.

(ii) In river-water from the Folkestone beds than in river-water from the Sandgate and Hythe beds.

(iii) In river-water from Gravel, Sand, and Clay formations than in river-water from the Chalk.

7. Mud from unpolluted rivers contains a considerable amount of oxidisable matter and may have a deoxygenating effect upon the water from 3 to 35 times as great as that of a good standard sewage effluent. The actual volume of the superficial deoxygenating mud relative to that of the supernatant water being small, however, muds from clean rivers will in general have less polluting effect than an effluent.

8. Considerably lower or higher results in the dissolved oxygen absorption test may be obtained when tap-water from the Gravel and Upper Bagshot

Sands is used for dilution than when the river-water into which the effluent actually drains is employed. Sometimes however there is close agreement between the two sets of figures.

9. Lower results are often obtained when hard calcareous tap-waters are used for dilution instead of the river-waters. Occasionally however the results are higher, while sometimes there is no appreciable difference noticed.

10. There may sometimes be close agreement in the dissolved oxygen absorption figures of an effluent diluted respectively with river-water and distilled water.

In many cases however, *e.g.* river-water from the Gravel and Upper Bagshot Sands, Lower Greensand, and Chalk, there is a considerable difference, and oxidation may go on more slowly or more rapidly in the river-water than in distilled water; the river-water thus having an inhibitory or accelerating action as the case may be.

In the case of river-water from the Gravel and Upper Bagshot Sands, and the Chalk, the *inhibitory* action upon the processes of oxidation may be especially marked.

11. The main conclusion to be drawn from the results summarised in 6–10 is that in the standardisation of sewage effluents the river-water into which the effluent is actually discharged must be used for dilution in Winkler's dissolved oxygen absorption test, unless it can be conclusively shown by several experiments that reliable results can be obtained by employing a more convenient diluting medium, *e.g.* another river-water, tap-water, or distilled water.

12. It is very likely that the oxidation processes in artificial methods of sewage purification may be affected by the nature of the water-supply, and by surface water entering the drainage system. Both hard and soft tap-waters may exert a very considerable inhibitory action in laboratory experiments. It is recommended therefore that allowance for possible inhibitory factors be always made in preparing new schemes of sewage purification, so that additional filtering area can be subsequently added, if necessary, with the minimum of trouble and expense.

13. The oxidation by permanganate or Tidy Test, unlike the dissolved oxygen absorption test, does not appear to be affected by the presence of waters of different geological source.

A consideration of the facts leads to the conclusion that the permanganate test is more suitable for the chemical examination of water supplies, while the dissolved oxygen test is more suitable for the standardisation of sewage effluents.

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XXXV. ON THE DIGESTIBILITY OF COCOA BUTTER. PART I.

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There is a considerable literature dealing with the value of cocoa as a food, and a number of observers describe experiments, carried out both on man and animals, to test the physiological utilisation of the fats and proteins contained in it. In spite of the fact that the fat is of high melting point, and is stated to be resistant to enzyme action *in vitro*, it appears, when cocoa itself is eaten, to be well absorbed and utilised. The proteins are on the whole less available, but in the majority of the experiments recorded they show a fairly satisfactory utilisation.

H. Cohn [1895] in experiments carried out upon himself found that over 95 % of the fat was absorbed, but only about half the protein. R. O. Newmann [1906] in prolonged experiments got favourable results, and found that, when moderate quantities were used, the utilisation of the fat was about 90 %. Similar results were obtained by V. Gerlach [1907]. L. Pincussohn [1907], who added 35 g. of cocoa per day to the diet of a number of subjects, found that the fat absorption was very good, in some cases as high as 98 %. The protein absorption was, however, variable, ranging from 60 to 90 %, but was best in preparations containing a low percentage of fat. N. Zuntz [1890] in the case of chocolate found that 95 % of the fat was utilised. None of these observers noticed any ill-effects upon health as the result of taking for considerable periods quantities in excess of what would be consumed in ordinary life.

Until recently there were comparatively few experiments recorded on the feeding of the actual fat contained in cocoa-beans, namely cocoa butter. Zuntz in the above-mentioned paper, in a very extensive series of observations, found that dogs fed with cocoa butter absorbed about 90 %; a utilisation very nearly as good as in the case of bacon fat. Langworthy and Holmes [1917] published a paper on the digestibility of some vegetable fats, and amongst these fats cocoa butter was included. These experiments were conducted by similar methods to those that were employed in a previous investigation [1913] on animal fats. A basal ration, supplying a minimum of fat, composed of wheat biscuits, oranges, sugar and tea, or coffee if desired, was supplemented by a blancmange or corn-starch pudding, in which was incorporated the vegetable fat under consideration. This diet was fed to normal young men, in

good health and moderately active, for periods of *three* days. Weighings were made of nett amounts of food eaten and faeces excreted, and samples of both food and faeces were analysed to determine the percentages of protein, fat and carbohydrate which were actually digested.

The digestibilities of olive oil, cotton seed oil, pea nut oil, coconut oil, sesame oil and cocoa butter were in this way compared. In 11 experiments using different amounts of cocoa butter, the average percentage utilisation was 89·6. The figures however were rather variable, running from 79·1 % to 95·5 %.

The fat-carrying blancmange was *not relished in the cocoa butter experiments* as it was in the case of the other fats, for the amount eaten was only enough to supply 51 g. of cocoa fat per man per day during the first eight experiments. This quantity did not cause any decided digestive disturbance so far as was noted. The subjects reported a "loss of appetite," but in all other respects considered that their physical condition had been normal. In later tests the subjects were urged to eat more of the blancmange containing the cocoa butter. As a result of eating the larger quantities, an average of 109 g. per day, undesirable physiological derangements were experienced. The effects were so pronounced that one subject discontinued the diet at the end of the seventh meal. "Loss of appetite," "headache," "loss of ambition," "nausea" and "sleeplessness" were conditions reported, which, the authors consider, indicate that cocoa butter in quantity had an effect not noted in the case of the other vegetable fats studied. Though the exact limit of tolerance was not determined, to judge by the experiments made in the laboratory, the maximum amount of cocoa butter that can be consumed daily without decidedly unpleasant effects lies between 51 and 109 g.

The digestibility of the carbohydrate and protein did not seem to be influenced by the digestibility of the fat. In the experiments in which large quantities of the fat were fed, the faeces contained comparatively large quantities of fat—in one experiment as much as 37 % of the dry weight. In this case they state that an odour suggesting that of cocoa butter could be clearly detected.

This latter observation is however misleading, as a smell of cocoa is in our experience a normal constituent of the smell of all faeces. Dry faeces, after extraction with ether, retain this component of the smell in a marked degree, and it has nothing to do with the nature of the diet.

Messrs Langworthy and Holmes give the following figures for the coefficients of digestibility of the oils examined. In arriving at these numbers an allowance for metabolic products is introduced.

Olive oil	97·8
Cotton seed oil	97·8
Pea nut oil	98·3
Coconut oil	97·9
Sesame oil	98·0
Cocoa butter	94·9

With regard to cocoa butter they conclude that in view of the unsatisfactory utilisation of this fat and the accompanying physiological disturbances, the advisability of the continued daily use of cocoa butter in large amounts would appear questionable.

Nature of the present investigation.

The object of the experiments detailed below was to arrive at an opinion as to whether cocoa butter could be utilised with economy as part of the fat supply of the population without detrimental results to health.

In studying the digestibility of a single food, experience has shown that it is advisable to incorporate it in a simple mixed diet, since the ordinary individual is so accustomed to a mixed diet that, no matter how palatable a simple food may be at first, it soon becomes distasteful. It is also essential that the diet in question should be palatable, and we think it desirable that no constituent of the diet should be very largely in excess of the physiological balance. This was obviously not the case in the American experiments in which large quantities of cocoa butter were fed. The diets made use of by the sub-committee of the Royal Society in their experiments on the digestibility of breads made from different kinds of flour [1918] seemed very suitable for the purpose, and in order to save time it was decided to utilise the material accumulated in these experiments as controls.

The Diets consumed.

The details of the control diets have been published in the report by the Food (War) Committee of the Royal Society on the Digestibility of Breads [1918]. Approximately the daily diet consisted of

Bread	800–1000 g.
Minced meat	50 „
Butter	50 „
Jam	100 „
Milk	600 cc.
Cheese	50 g.
Sugar	30 „
Tea	*ad lib.*

In the tables in this paper the control diets, the fat content of which has not hitherto been published, are referred to as A, B, and C. In A the bread was made from 80 % milled flour, in B from 90 % milled flour and in C from 80 % milled flour mixed with 20 % maize. Almost the same diet was used in the cocoa butter experiments, substituting 50 g. of cocoa butter for the 50 g. of butter. The bread used in these latter experiments was the ordinary bread of the shops, and the daily ration was about 850 g. It was not possible to use the special breads of the control diets, but the results of the bread experiments warrant the assumption that the use of ordinary shop bread would have little

or no influence on the results. However, to obviate this as far as possible each subject was instructed to purchase his own bread.

Probably the results are more strictly comparable with diet C, the bread of which was made from mixed cereals, since the bread supplied to the public at the time of the cocoa butter experiments was of this nature. The cocoa butter used was the ordinary fat sold under that name.

It was rather hard and brittle, pale yellow in colour and possessed when fresh a distinct flavour of cocoa, which however rapidly diminished in intensity on keeping even in a closed vessel.

It consisted almost entirely of pure fat, and the constants given below are well within the limits of variation given in the literature.

Moisture	0·17 %	Melting point	33·5 ° C.
Ash	0·059 „	Acid value	1·98
Unsap. matter	0·907 „	Sap. value	194
Sterols	0·171 „	Reichert-Meissl value	0·84
		Iodine value	37·3

Subjects used in the experiments.

An account of the eight subjects, four in Cambridge and four in London who had the control diets is given in the above-mentioned report. Out of the four subjects, previously experimented on in London, two only, viz. E. and B., were still available for the cocoa butter experiment, the others having been called to the colours. We were only able to get one additional subject—P.

Individual	Age	Wt in kilos	Occupation and state of health
E.	36	55	Laboratory assistant. Delicate health
B.	34	65	Laboratory attendant. Athlete and active worker
P.	18	64	Laboratory attendant. Active and in good health

E. and B. were therefore the only subjects who had both the control and the cocoa butter diets.

Experimental methods.

The general procedure was exactly the same as that detailed in the Royal Society Report on the Digestibility of Breads, the diet being consumed for a period of ten days, the six significant days being the 4–9th inclusive. Accurate accounts of the weights of food eaten were kept and analyses of each food were made. On the fourth, sixth and ninth days a determination of the nitrogen in the urine was also made.

As before, the collection of faeces began one day later and continued one day longer than the days of the diet.

Methods of analysis.

The coefficients of digestibility given in Langworthy and Holmes' paper referred to above were obtained in the customary way by analysing the food eaten and the faeces excreted during an experimental period. For this purpose

the total ether extract of the faeces was taken to represent the actual quantity of undigested fat—the unavailable residual of the fat eaten during the experimental period. That this assumption is far from true is apparent from a study of the composition of the faeces. It is well known that the faeces contain not only undigested food, and undigested residues of food, but also other waste materials, usually grouped under the somewhat ambiguous term metabolic products, some of which are soluble in ether. The ether extract, usually considered as fat, consists of true fat, fatty acids, and unsaponifiable matter. The unsaponifiable matter consists mainly of a variety of alcohols of the sterol group with a small proportion of fatty alcohols of lower molecular weight such as cetyl alcohol, etc. This unsaponifiable matter is partly metabolic in origin and is partly contained in the foodstuff. The ratio of true fat to fatty acids is variable, but as a rule the quantity of true fat is small and is often negligible. Faeces also contain salts of fatty acids, or soaps which are insoluble in ether.

It was therefore considered necessary to submit both the food and faeces to a more detailed analysis than is usually the case.

The faeces were analysed in the following manner. A weighed portion of the dried faeces was subjected to a prolonged extraction (not less than a week) in a Soxhlet apparatus with ether, and the ethereal solution was made up to known volumes. Aliquot portions were then respectively titrated with standard alcoholic soda, and evaporated to dryness and weighed. In the latter estimation care must be taken not to overheat. The rest of the ethereal solution was then mixed with an alcoholic solution of sodium in considerable excess and allowed to stand 48 hours. The precipitated soaps were then filtered on the pump and thoroughly washed with ether. The ethereal filtrate and washings were then shaken with alkaline water and finally with distilled water until quite free from soap. The ethereal solution of unsaponifiable matter thus obtained was made to known volume and an aliquot portion evaporated and weighed. Estimations of the sterols, precipitable by digitonin, in the unsaponifiable residue were also made, but these values will form the subject of a separate communication. In order to estimate the fatty acids in the form of soaps, the faecal residue after extraction with ether was moistened with an alcoholic solution of hydrochloric acid, dried and again extracted with ether. In this case the acids were not estimated by evaporating the ethereal solution, as it was thought that the more soluble fatty acids being stronger than those of high molecular weight would mainly be found in these soaps. The ethereal extract was therefore exactly neutralised by a standard alcoholic soda, evaporated to dryness and weighed.

Fat in the foods was determined by extraction with ether in the usual way, and the unsaponifiable matter and sterols estimated as described. In the case of bread and meat the ether extraction was preceded by extraction with boiling alcohol, as it is well known that in such substances the extraction of fat by ether alone is imperfect, however long the extraction may be prolonged. The results are given in the following tables.

Table I.

The percentage availability of the nitrogen of Diet D per day and also the nitrogen balance per day.

		E.	B.	P.	
Nitrogen	Intake in food ...	16·51	20·35	19·87	
	Excreted in urine ...	13·91	13·47	12·99	
	„ in faeces ...	2·12	2·80	2·78	
	Total ...	16·03	16·27	15·77	
	Nitrogen absorbed ...	14·39	17·55	17·09	
	Balance	+ 0·48	+ 4·08	+ 4·10	
	Percentage utilisation	87·14	86·2	86·0	Average 86·4

Average percentage utilisation in Diet A, 89·4; Diet B, 87·8; Diet C, 89·3.

Table II.

Diet A.

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	98·93	97·28	99·41	92·79	155·28	92·53	122·08	139·93
	Excreted in faeces	3·52	3·66	4·33	3·11	4·94	1·63	2·47	2·32
	Difference	95·41	93·62	95·08	89·68	150·34	90·90	119·61	137·61
	% utilisation	96·44	96·24	95·65	96·65	96·81	98·23	97·98	98·34
		Average = 96·24				Average = 97·84			
		Average = 97·04							
True fat in food	Intake in food	96·62	94·96	97·01	90·73	150·94	89·75	119·58	137·22
Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Excreted in faeces	5·08	2·76	3·37	2·34	7·14	2·29	3·88	2·48
	Difference	91·54	92·20	93·64	88·39	143·80	87·46	115·70	134·74
	% utilisation	94·74	97·07	96·53	97·42	95·27	97·45	96·76	98·19
		Average = 96·44				Average = 96·92			
		Average = 96·68							

Table III.

Diet B.

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	101·83	94·50	107·40	105·40	155·59	113·69	107·71	144·70
	Excreted in faeces	2·77	—	1·74	2·13	3·38	3·71	2·63	4·97
	Difference	99·06	—	105·66	103·27	152·21	109·98	105·08	139·73
	% utilisation	97·28	—	98·38	97·98	97·83	96·74	97·56	96·57
		Average = 97·88				Average = 97·18			
		Average = 97·48							
True fat in food	Intake in food	99·16	92·23	104·47	102·70	151·06	110·31	105·05	140·55
Fat excreted in faeces as fat, fatty acids and fatty acids in form of soap	Excreted in faeces	4·41	—	2·52	3·78	5·38	5·77	3·53	6·69
	Difference	94·75	—	101·95	98·92	145·68	104·54	101·52	133·86
	% utilisation	95·55	—	97·59	96·32	96·44	94·77	96·64	95·24
		Average = 96·49				Average = 95·77			
		Average = 96·08							

Table IV.

Diet C.

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	98·23	97·75	99·01	91·03	167·30	109·50	109·70	163·50
	Excreted in faeces	3·73	4·17	3·49	3·57	4·06	2·50	2·41	4·12
	Difference	94·50	93·58	95·52	87·46	163·24	107·00	107·29	159·38
	% utilisation	96·20	95·73	96·48	96·08	97·57	97·72	97·79	97·48
		Average = 96·12				Average = 97·64			
		Average = 96·88							
True fat in food	Intake in food	96·85	95·44	97·46	89·76	163·30	106·10	107·24	159·53
Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Excreted in faeces	4·36	6·54	3·72	3·59	4·06	2·50	4·12	2·41
	Difference	92·49	88·90	93·74	86·17	159·24	103·60	103·12	157·12
	% utilisation	95·50	93·15	96·18	96·00	97·51	97·64	96·16	98·49
		Average = 95·20				Average = 97·45			
		Average = 96·32							

Table V.

Diet D.

		London subjects		
		E.	B.	P.
Total ether extract	Intake in food ...	109·00	105·73	105·53
	Excreted in faeces ...	5·41	6·66	6·66
	Difference	103·59	99·07	98·87
	Percentage utilisation	95·04	93·70	93·68
		Average = 94·14		
True fat in food	Intake in food ...	107·77	104·40	104·25
Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Excreted in faeces ...	6·27	7·56	7·62
	Difference	101·50	96·84	96·63
	Percentage utilisation	94·18	92·76	92·69
		Average = 93·21		

Table VI.

Comparative table showing average percentage fat utilisation in different diets.

	Diets			
	A	B	C	D
Total ether extract	97·04	97·48	96·88	94·14
True fat in food, and excreted in faeces as neutral fat, fatty acids and fatty acids in form of soap	96·68	96·08	96·32	93·21

Health of the subjects.

All the subjects found the cocoa butter palatable, and were quite content with the diet. They suffered no appreciable change in weight during the experimental period. Their excreta were normal in appearance, and no apparent digestive trouble was noted. Their health was normal throughout.

Discussion of results.

Assuming that the bread sold to the public at the date of the experiments (1918) is comparable with that used in the diets A, B and C, it will be seen from Table I that in the case of the cocoa butter diet D there is a small but definite decrease in the utilisation of nitrogen, a result in agreement with observations recorded by various observers in experiments with cocoa.

The average percentage utilisation of fat in the cocoa butter diet D, whether judged by the total ether extract or by the true fat, is lower than the average values in the butter diets A, B and C, but the difference is not very marked, and the figures come within the limits of variation of A and C. The utilisation from this point of view must be regarded as satisfactory.

It will be noticed that the figure we obtained for the percentage utilisation of ether extract in diet D (94·14) is considerably higher than the figure 89·6 given by the American observers, though it comes within their limits of variation 79·1 to 95·5. It must be remembered however that diet D contained in addition to the cocoa butter a considerable amount of ordinary fat present in the meat, milk and cheese. That this slightly lower average utilisation is real and not merely the result of the smaller number of subjects eating diet D, and indicates that the cocoa butter is less digestible than ordinary butter, is shown by a consideration of the composition of the fatty matter of the faeces.

If we knew the average molecular weight of the acids excreted in the faeces on the various diets, it would be possible from the data we obtained to calculate for each individual the ratio of the fatty acids to unsaponified neutral fat in the faeces, since estimations were made on the ether extracts of (*a*) total solids by evaporation, (*b*) unsaponifiable matter, (*c*) free acids by titration with standard alkali: the difference between (*a*) and ($b + c$) would give the neutral fat. We do not know the average molecular weights of the acids in question, and furthermore the values for total solids may be slightly lower than the true values owing to loss of volatile acid on evaporation of the ether. We could not therefore get an accurate quantitative relation between the fatty acids and neutral fats in the faeces, but by assuming that the fatty acids were all stearic it can be shown that there is a marked difference between the composition of the faeces A, B and C on the one hand, and D on the other. This will be clear from the following figures for the two subjects who had all four diets.

Table VII.

Weights per cent. of dry faeces.

Diet	Individual	Total ether extract	Unsaponifiable matter	Real fatty matter	Approximate amount of fatty acid reckoned as stearic acid	Neutral fat by difference
A	E.	13·13	3·95	9·18	11·71	Probably nil
	B.	14·36	3·58	10·78	10·31	0·47
B	E.	6·99	2·40	4·59	8·37	Probably nil
	B.	5·50	1·72	3·78	6·28	,,
C	E.	12·07	3·30	8·77	"15·9"	,,
	B.	13·14	3·25	9·89	10·1	,,
D	E.	15·18	1·96	13·22	1·41	11·81
	B.	15·48	1·80	13·68	4·05	9·63
	P.	14·07	0·90	13·17	4·54	8·63

It will be seen that whereas in the diets A, B and C the fatty matter of the ether extracts was mainly acids with little or perhaps no neutral fat, in the case of diet D the fat of the ether extract was mainly neutral fat. This clearly indicates that some small portion of the cocoa butter passed the intestinal tract unchanged.

More light would however be thrown on this question by a knowledge of the actual components of the fats used in the various diets, especially cocoa butter which differs markedly from animal fats and the more liquid vegetable fats, and of the fatty matter in the faeces produced. Experiments are being made on the separation of the acids from these various substances by fractional distillation of their methyl esters, with a view to the determination of the molecular weights, iodine values, etc. of the different fractions. These results are nearing completion and we hope shortly to be able to publish them.

Qualitative experiments.

In order to get some idea of the tolerance for cocoa butter, and to find whether physiological disturbances similar to these noted by the American observers were produced when it was fed in excessive quantity with ordinary diet, four workers in the laboratory volunteered to try and eat three to four ounces per day for four days or so.

The four subjects were F. W. F. a chemist, W. L. S. a physiologist, W. G. R. a cadet of the O.T.C., and G. T. W. a cadet of the O.T.C.

They report as follows:

G. T. W. When I first started eating cocoa butter I did not enjoy it at all —that was eating about four ounces per day—but after the first two days I quite liked it, and found it most palatable when eaten with bread and jam. It has had no effect on me at all. The amount eaten was 1 lb. in five days.

W. G. R. Quantity eaten per day three to four ounces. At first the cocoa butter was unpleasant, giving rise to a bilious feeling, but later, when taken slightly warm with salt, this was not noticeable.

So long as it was eaten with some strong flavouring it made a fairly good substitute for butter, but was not pleasant when eaten alone with bread.

In spite of the fact that the amount taken was far in excess of my usual butter allowance, no ill effects were experienced.

W. L. S. I could not stomach much of your cocoa butter. I managed one ounce the first day, and was troubled by eructations. It also proved distinctly laxative. On the second day I could only take ½ ounce and since then have taken none. It did not appear to be a very good sample[1].

F. W. F. I deliberately ate even larger quantities of cocoa butter than those given by the American observers, but without experiencing any of the unpleasant physiological effects they mention. The weights taken for the five days were as follows: 56, 237, 201, 102 and 135 g. These quantities were very far in excess of my ordinary fat ration, and were naturally unpalatably large. The faeces showed the high value, for the ether extract, of 24·3 g. % of dry weight.

General conclusions.

1. Cocoa butter is rather less digestible than butter, but the experiments recorded show satisfactory utilisation.

2. Beyond the slight laxative action observed in the case of some of the subjects who consumed large quantities of the cocoa butter, no undesirable physiological effects were noticed.

3. Our experiments show no indication whatever that cocoa butter is a "slow poison," and there can be no doubt that this substance could be used to supplement the fat need of the population with safety. This conclusion is in agreement with general experience, since large quantities of this fat must have been consumed with impunity in the various chocolate preparations on the market—which may contain as much as 20 % of fat.

This work was undertaken at the request of the Food (War) Committee of the Royal Society, by whom the expenses were defrayed.

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[1] The same cocoa butter was supplied to each volunteer, and its constants were normal.

XXXVI. A NEW METHOD FOR PREPARING ESTERS OF AMINO ACIDS. COMPOSITION OF CASEINOGEN.

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In a preliminary paper [Foreman, 1912] reasons were given for attempting to improve the methods commonly in use for separating the products of protein hydrolysis, and a new method for preparing the esters of the amino acids was briefly described.

The method has been applied to the products of two further hydrolyses of caseinogen with the following objects in view:

A. Hydrolysis by sulphuric acid. To determine the distribution of the nitrogen in the various fractions, and to test the completeness of the esterification.

B. Hydrolysis by hydrochloric acid. To determine the effect of the process upon the yields of the monamino acids, and to endeavour to throw some light upon the nature of the unidentified substances making up the deficit in protein analysis.

Before describing the experiments, it will be advisable to set forth briefly the steps involved. Full details of the working will be found in the account of Hydrolysis *B*.

(*a*) The amino acids are converted into their dry lead salts.

(*b*) The lead salts are suspended in absolute alcohol, and the esterification brought about by saturating with dry hydrochloric acid gas.

(*c*) The free hydrochloric acid is removed, partly by reducing the liquid to half its bulk at 40° and 15 mm., and the remainder by the addition of absolute alcohol saturated with dry ammonia gas.

(*d*) Alcohol is removed by evaporation at 40° and 15 mm., and the ester hydrochlorides taken up in dry chloroform.

(*e*) The esters are liberated from their hydrochlorides, and any remaining traces of esterification water removed by shaking the chloroform solution with anhydrous baryta.

(*f*) The chloroform is evaporated at 40° and 15 mm., and the esters taken up in anhydrous ether.

Hydrolysis A.

Nitrogen distribution. 320 g. "Hammersten casein," containing 45·056 g. N equal to 288·4 g. pure caseinogen calculated on the basis of 15·62 % N, was hydrolysed by boiling for 48 hours with three parts of sulphuric acid and six parts of water. The sulphuric acid was removed by means of baryta before making the lead salts.

The following table shows the percentages of the total nitrogen found in the fractions:

In the crude tyrosine removed	1·98
In the barium sulphate precipitate after very thorough washing	7·13
In the aqueous solution of amino acids	88·38
Loss	2·51
	100·00

From the aqueous solution of amino acids:

Ammonia lost when making the lead salts (calculated from the percentage for caseinogen given by Osborne and Guest [1911])	8·48
In the undissolved lead oxide and hydroxide	1·36
In the dry lead salts	77·08
Loss	1·46
	88·38

From the lead salts:

In the washed lead chloride removed after the esterification	1·63
In the filtrate containing the ester hydrochlorides	75·02
Loss	0·43
	77·08

The baryta residue obtained when the esters were liberated from their hydrochlorides contained 13·42 %, and a small quantity of esters soluble in chloroform but insoluble in ether 2·06 % of the total N. When removing the ether by distillation *in vacuo* from about half the ester solution, the flask was slightly fractured by accident and water gained access to the solution. The esters were recovered as far as possible by ether extraction, but small losses undoubtedly occurred. The nitrogen determinations were therefore discontinued at this stage. After removing the ether the esters weighed 244·7 g.

The ester process was not applied a second time to the material taken from the chloroform solution of the ester hydrochlorides by the anhydrous baryta. The quantity of esters obtained therefore represents the yield of a single preparation.

The esters were fractionally distilled in the usual way, and the fractions partially worked up. The quantities of amino acids separated, so far as these operations were carried, are given in the following table:

Amino acids	Total amounts obtained from 288·4 g. caseinogen. Obtained by one esterification
Glycine	0·5 g. triglycylglycine ester
Alanine, leucines and valine	52·09 g.
Proline	20·12 g.
Phenylalanine	8·96 g. of the hydrochloride
Aspartic acid	4·2 g.

The proline was estimated by van Slyke's method [1911].

The 8·96 g. phenylalanine hydrochloride represents the first crop of the crystals. The mother liquor would have yielded a further quantity. Phenylalanine ester was undoubtedly extracted to some extent by the water which gained admission to the ethereal solution of the esters by accident (p. 379).

The aspartic acid was separated as barium aspartate from the highest boiling ester fraction.

The 0·5 g. triglycylglycine ester separated from Fraction I while it was standing in the ester condition in a corked bottle for about three weeks. The substance was easily soluble in water, alkaline to litmus, and gave a pronounced biuret reaction. When its melting point was taken, it commenced to turn brown at 200°, sintered on further raising the temperature, blackened and finally decomposed at 267°. Fraction I always contains ether. The conditions were therefore identical with those under which Curtius [1904] prepared his biuret base, triglycylglycine ester. The composition, properties and reactions of the substance were also in agreement. Found 20·6 % N; calculated for triglycylglycine ester 20·44 % N.

The efficiency of the esterification method.

The dry lead salts weighed 558 g. and contained 50·27 % Pb. The lead chloride removed by filtration after esterifying, dried at 100°, weighed 335·7 g. The filtrate was found to yield a precipitate on adding absolute alcohol, which settled to the bottom in the form of a brown syrup. Extraction with a little cold water gave a brown solution containing 2·727 g. N leaving 36·2 g. lead chloride. The brown solution invites further investigation. The total weight of dry lead chloride recovered was therefore 371·9 g., which corresponds to 277 g. lead. The lead salts contained 280·5 g. lead. The lead salts were therefore practically completely transformed in the esterification.

Hydrolysis B.

YIELDS OF MONAMINO ACIDS. INVESTIGATION OF THE RESIDUES.

In the previous sulphuric acid hydrolysis, no less than 7 % of the total N was retained by the washed barium sulphate precipitate formed when the acid was removed. In order to avoid the effect of this loss upon the yields of amino acids, this hydrolysis was brought about by means of hydrochloric acid. Glutamic acid was removed in the form of its hydrochloride, as far as possible, before the esterification.

320 g. of the same sample of "Hammersten casein" as used in Hydrolysis *A* (= 288·4 g. pure substance) was hydrolysed in the usual way by boiling with hydrochloric acid for 48 hours.

Removal of glutamic acid as the hydrochloride. The liquid arising from the hydrolysis was evaporated on the water-bath to a volume of about 700 cc., saturated with hydrochloric acid gas, and left in the ice chest. The crystals

were filtered off on an asbestos pad, and washed several times with aqueous hydrochloric acid saturated with the gas at 0°. The crude hydrochlorides thus obtained weighed 88·1 g. after drying to constant weight. This material was taken up with water, the solution decolorised by warming with animal charcoal, reduced to about 300 cc., and saturated with hydrochloric acid gas. The perfectly white crystals were filtered off on asbestos, and washed many times with ice cold saturated aqueous hydrochloric acid. The free hydrochloric acid was removed in a desiccator over strong potash. 57·1 g. resulted after drying to constant weight at 100°. The filtrate yielded a further 0·16 g. of the hydrochloride when reduced in bulk and again saturated. The total weight was therefore 57·26 g., which corresponds to 45·87 g. glutamic acid. Portions weighed out from the whole specimen gave the following results on analysis:

	Found	Calculated
	%	%
C	32·61	32·70
H	5·80	5·45
N	7·64	7·62
Amino N (van Slyke)	7·74	7·62

It will be noted that the recrystallised material only accounted for about two-thirds of the crude dry hydrochlorides.

Preparation of the lead salts. The filtrate from the recrystallised glutamic acid hydrochloride was added to the main hydrolytic liquid, and hydrochloric acid removed as far as possible by evaporation on the water-bath. A current of steam was then passed through the heated fluid for some time to remove further hydrochloric acid. The volume was made up to 2 litres with water, a current of steam passed through, and 100 g. precipitated lead hydroxide added. The steaming was continued for a few minutes, and the hot solution filtered by suction. The insoluble material was dark in colour, and contained the greater part of the humin matter. 400 to 500 g. ordinary litharge was added to the filtrate in portions during a further 45 minutes' steaming. The excess of litharge was filtered off rapidly by suction, and washed with hot water.

The preliminary treatment with lead hydroxide was introduced because the compound formed with the humin matter remains in suspension, and can be easily filtered off. When litharge was used from the commencement, however, the humin compounds tended to collect at the bottom in the form of a troublesome cake. The use of litharge after lead hydroxide appears to be necessary for the complete transformation of the amino acids into lead salts, and a good excess should be added as the bigger particles are not readily dissolved.

The solution containing the lead salts was evaporated on the water-bath to a volume of about 500 cc. and cooled. The semi-solid mass of salts which resulted was placed on the water-bath and stirred until it became too viscous. After standing in the steam-oven all night and then being cooled the material can be easily reduced to a powder. The grinding however is quite unnecessary,

as the dry cakes will esterify readily when treated in the manner to be described. The dry lead salts weighed 475 g. The undissolved lead hydroxide and litharge contained 3·1 % of the total N of the 320 g. caseinogen.

Esterification. The lead salts were suspended in 1500 cc. absolute alcohol, and saturated with dry hydrochloric acid gas. The liquid was then cooled by means of a freezing mixture of ice and salt, and again brought to saturation. The lead chloride was filtered off and washed with absolute alcohol.

Removal of the free hydrochloric acid. The greater part of the free hydrochloric acid was removed by reducing the volume to one-half at 40° and 15 mm. The fluid was transferred to an enamelled jug, and the flask washed out with absolute alcohol. The jug was surrounded by a freezing mixture of ice and salt, and the remaining free hydrochloric acid removed by means of a saturated solution of dry ammonia gas in absolute alcohol, which was added slowly, with continual stirring, until the liquid remained only faintly acid. The further addition of alcoholic ammonia would liberate esters from their hydrochlorides, and the fluid would darken in colour. It was considered advisable, however, to stop at the faintly acid stage in order to avoid loss which might result from decomposition of the free esters in this as well as subsequent operations. By the means described the free acid was eliminated without involving the formation of water. The ammonium chloride was filtered off by suction, and washed thoroughly with absolute alcohol.

Removal of the alcohol. The alcohol was removed as completely as possible by evaporation at 40° and 15 mm. The specific gravity of the distillate was found to be higher than that of absolute alcohol. Assuming this increase to be due to the presence of water, the distillate contained a quantity which corresponded very well with the calculated amount of water produced during the esterification. The content of lead in the lead salts of the previous sulphuric acid hydrolysis provided a basis for making this calculation, after an allowance had been made for the glutamic acid removed in the case of Hydrolysis *B*.

The ester hydrochlorides taken up in dry chloroform. The syrup was taken up in dry chloroform, in which the ester hydrochlorides dissolved readily. A little ammonium chloride, which had been retained in solution by the alcohol, was filtered off and washed with chloroform. The total amount of ammonium chloride formed from the free hydrochloric acid was about 250 g.

Liberation of the esters from their hydrochlorides. The chloroform solution of the ester hydrochlorides occupied a volume of 1000 to 1500 cc. 300 to 350 g. anhydrous baryta were added in successive portions of about 30 g., and the mixture well shaken after each addition. After all the baryta had been added, the mixture was allowed to stand for two minutes, by which time the temperature at the bottom where the solid material had settled had risen to about 35°. A temperature of about 30° throughout the whole fluid resulted on further shaking. The baryta by this time had become granular, and settled instantly the shaking was discontinued. The mixture was cooled under the tap, and a thermometer placed so that its bulb was covered by the solid material at the

bottom. After standing two or three minutes, a rise of 2° was recorded. The mixture was shaken again, cooled, and the test repeated, but no further increase of temperature resulted. It was concluded therefore that the reactions were complete. It may be found sometimes, however, that the clear fluid contains a little chloride at this stage. In this case the mixture should be allowed to stand, with an occasional shake, until no chloride remains. In one experiment the addition of a little more anhydrous baryta was necessary before the last traces of chloride could be removed. The solid material was filtered off by suction and washed thoroughly with dry chloroform. The chloroform was removed from the filtrate by evaporation at 40° and 15 mm., and the esters shaken with anhydrous ether. Practically the whole dissolved immediately, only a very small quantity of syrup which collected on the sides of the flask remaining insoluble. The latter was found to contain substances precipitated by phosphotungstic acid after hydrolysis. The esters weighed 235·9 g.

Recovery of a second portion of esters. The baryta residue obtained when the esters were liberated from their hydrochlorides contained 9·6 g. N. This was suspended in water, and the barium all converted into sulphate at 40° by adding a slight excess of dilute sulphuric acid. After heating for some time on the water-bath, the barium sulphate was removed and washed. The solution was steamed with litharge in the manner already described, and 116 g. dry lead salts obtained. Esterifying these lead salts as before, using equivalent amounts of the various reagents, only 25·6 g. of esters resulted.

The two lots of esters, together amounting to 261·5 g., were combined and distilled in the apparatus described by Fischer and Harries [1902]. For reasons which will be explained later (p. 393) it is regretted that these two quantities of esters were not investigated separately.

Fractional distillation of the 261·5 g. esters.

Fraction	Temp. of bath °C.	Temp. of vapour °C.	Pressure mm.	Weight of fraction, g.
I	Up to 88	Up to 65	11	28·07
II	88 to 110	65 to 84	10	73·71
III	110 to 132	84 to 94	10	20·00
IV	152 to 165	100 to 125	10	13·57
Distillation residue	—	—	—	82·03
In liquid air condenser	—	—	—	10·45
			Total	227·83

Fraction IV did not commence to distil over until the temperature of the bath was 152° and of the vapour 100°. A very slow current of carbon dioxide, too small to increase the pressure, was passed through the liquid during the distillation. The fractions were thus assisted in their passage to the condenser, and air was excluded. An accident to the Dewar's flask containing the liquid air supply just before the reduction of pressure was required prevented the usual fractions at 0·5 mm. from being obtained. The distillation was continued

at 10 mm. as described, and a very satisfactory separation of the fractions containing glycine, alanine, valine, leucines and proline was effected.

Phenylalanine. Fraction IV was treated with water and extracted with ether in the usual way, and the ether-soluble ester evaporated to dryness with hydrochloric acid. The crystals obtained were dissolved in water, decolorised with animal charcoal, and recrystallised from hydrochloric acid. The specimen was washed thoroughly with saturated aqueous hydrochloric acid, and the free acid removed in a desiccator over potash. This fraction of the phenylalanine hydrochloride, dried completely at 100°, weighed 1·36 g. It contained 7·07 % N (Kjeldahl).

The distillation residue was treated in exactly the same way as Fraction IV, yielding 11·71 g. of the dry recrystallised hydrochloride. This contained 6·87 % N; calculated for phenylalanine hydrochloride 6·95 % N.

The total quantity of hydrochloride separated was 13·07 g. equal to 10·70 g. phenylalanine.

The liquids from which these separations of the hydrochloride had been made were combined, and an attempt made to obtain a further crop of the hydrochloride. A little more was separated, but its content of nitrogen was 0·45 % too high. It was returned therefore, and the amino acids liberated by quantitatively removing the free as well as the combined hydrochloric acid. A total weight of 7·89 g. dry substance resulted, from which 0·25 g. tyrosine was separated, leaving 7·64 g. in which nothing was satisfactorily identified.

Glycine and alanine. The esters of Fraction I, obtained up to a temperature (vapour) 5° higher than usual, were hydrolysed by boiling with water, and the solution evaporated to dryness. After extracting proline in the usual way, the amino acids, dried at 100°, weighed 13·28 g. By fractional crystallisation from water, one fraction was obtained which melted at 250°, and contained 13·05 % N. This was sub-fractionated, but no portion contained a much higher percentage of N, and no alanine could be separated. The other original fractions gave much higher melting points, and judging by their nitrogen content consisted of mixtures of valine and leucine.

The mother liquor on evaporation yielded 6·58 g. dry amino acids, which melted at 239°, and contained 16·03 % N. This was examined for glycine by the picrate method of Levene and van Slyke [1912], and 2·38 g. glycine picrate melting at 200°, and decomposing at 202°, was separated. The picric acid was removed from the filtrate by means of *N* sulphuric acid and ether and the sulphuric acid by baryta. The amino acids recovered, dried at 100° C., weighed 4·77 g. This material was crystallised from water, leaving a small mother liquor which on evaporation yielded 1·45 g. dry substance melting at 238°, from which a further 0·756 g. glycine picrate with the correct melting point was separated. The two portions of glycine picrate, having identical melting points, were mixed, and the amino nitrogen in a portion weighed out from the whole quantity was determined by the nitrous acid method: found 7·33 % (corrected); calculated for glycine picrate 7·39 %.

The total quantity of the picrate thus separated from the amino acids of Fraction I was 3·136 g., which corresponds to 1·24 g. glycine.

The contents of the liquid air condenser, obtained when the esters were distilled, were freed from ether and hydrolysed by boiling with water. On evaporation 0·36 g. dry substance was obtained which melted at 239°, and contained 16·07 % N. This yielded 0·1 g. glycine picrate with the correct melting point, showing that losses were falling upon glycine ester during the distillation. The ether distilled from the ethereal solution of the esters before they were fractionated yielded by similar treatment 0·17 g. dry substance, from which no trace of glycine picrate could be separated.

The substance separated by crystallisation from the 4·77 g. amino acids recovered after removing the first lot of glycine picrate was submitted to analysis:

	Found	Calculated for alanine
C	40·82 %	40·45 %
H	7·97	7·86

It was concluded therefore that the 6·58 g. portion consisted of a mixture of 1·24 g. glycine and 5·34 g. alanine. This statement is supported by the fact that a mixture of glycine and alanine in these proportions would contain 16·15 % N, whereas 16·03 % was actually found.

Glycine from pure caseinogen. The examination of the first fraction obtained by distilling the esters prepared from the hydrolysis products of "Hammersten casein" by the method advocated in these pages revealed the presence of glycine. Evidence had been given which showed that losses of glycine occurred during the distillation owing to the low boiling point of its ester. The products of pure caseinogen therefore very probably contain considerably more than the 0·45 % isolated as the picrate. The specimen of picrate was excellent in quality. The 0·5 g. triglycylglycine ester separated in the previous hydrolysis (p. 380) provides confirmatory evidence.

If the absolute purity of "Hammersten casein" be doubted, it would be reasonable to suspect contamination with small quantities of the other protein constituents of milk. So far as I can ascertain, no glycine has been found amongst the hydrolytic products of lactalbumin, and the ester hydrolysis of lactoglobulin has not been done. A yield of 3·5 % glycine, however, has been obtained from serum globulin, which closely resembles lactoglobulin. Assuming lactoglobulin to contain 3·5 % glycine, and the 0·45 % glycine isolated from the products of caseinogen to be derived from lactoglobulin as impurity, the presence of 13 % of the latter in the caseinogen must be admitted. Emmerling [1888] states that milk contains not more than about 0·005 % lactoglobulin. Crowther and Raistrick [1916] however isolated 0·03 %, which is equivalent in quantity to only about 1 % of the caseinogen. The glycine separated therefore cannot be attributed to protein impurity.

Valine and leucines. The esters of Fractions II and III were hydrolysed in the usual way by boiling with water, and the solutions combined. The dry

amino acids obtained on evaporation were thoroughly extracted with absolute alcohol to dissolve proline, and dried at 100°. The following results were obtained on analysis: C 53·28, 53·22 %; H 9·67, 9·52 %; N (Dumas) 11·27, 11·28 %.

From these figures the proportion of leucine isomers to valine in the mixture was calculated as follows:

$$\frac{53{\cdot}28 - 51{\cdot}24}{54{\cdot}92 - 51{\cdot}24} \times 100 = 55{\cdot}4\ \%\ \text{leucine isomers.}$$

$$\frac{11{\cdot}27 - 10{\cdot}68}{11{\cdot}97 - 10{\cdot}68} \times 100 = 45{\cdot}7\ \%\ \text{valine.}$$

It will not be far from the mark therefore to conclude that the mixture consisted of 55 % leucine isomers and 45 % valine. To verify this further the lead method of Levene and van Slyke [1909] for the separation of these substances was employed. The lead salt of the leucines which separated was found to contain 44·64 % Pb. Calculated for lead leucine, 44·34 % Pb.

The leucine and valine were recovered from the lead precipitate and from the filtrate respectively, and their content of nitrogen determined.

Nitrogen in the leucine isomers:

0·1995 g.; (Kjeldahl) NH_3 neutralising 15·4 cc. *N*/10 acid, 10·80 % N. Calculated for leucine, 10·68 %.

Nitrogen in the valine:

0·2292 g.; (Dumas) 22·6 cc. N at 14·5° and 768 mm., 11·90 % N. Calculated for valine, 11·97 %.

The dry amino acids insoluble in alcohol obtained from the ester Fractions II and III, together with the small portions from Fraction I which showed a percentage of N between that of leucine and valine, amounted to 50·85 g. 55 % equals 27·97 g. leucines, leaving 22·88 g. valine.

Proline. The united alcoholic extracts of the dry matter obtained from ester Fractions I, II and III, already in such condition that the amino acids they contained would completely dissolve when well stirred in cold alcohol, were freed from alcohol, and the content of proline calculated from the non-amino nitrogen content according to van Slyke [1911]. The following results were obtained:

Total N	3·44 g.
Amino N	0·761
Proline N	2·679

This amount of proline N corresponds to 22·005 g. proline.

The material was dried to constant weight in the steam-oven, and weighed 28·05 g. It contained therefore 12·26 % total N, which is in accord with the experience of Osborne and Guest [1911]. A 2 g. portion was treated with phenyl isocyanate, and a moderate yield of the proline derivative was obtained. This was converted into the hydantoin, which melted at 143°.

Assuming all the non-amino N to be in the form of proline, 6·05 g. amino acids of the straight chain type were also present in the alcoholic extracts.

Aspartic acid. The esters of Fraction IV, from which phenylalanine ester had been extracted, were hydrolysed by boiling with baryta. A small quantity of barium aspartate separated which yielded 0·48 g. aspartic acid. This was converted into the copper salt which crystallised from plenty of water in the form of light blue needles. The barium was removed quantitatively from the filtrate from the barium aspartate, and the solution evaporated to dryness. After drying at 100°, this material weighed 3·71 g. By means of the titration method of Osborne and Liddle [1910] the presence of a further 0·51 g. aspartic acid was shown. The liquid which had been titrated was concentrated, and a small crystal fraction separated from dilute alcohol, melting at 245°. This substance was found to contain 39·39 % C, 7·82 % H, and 11·25 % N. An attempt to make a β-naphthalenesulphonic derivative gave no satisfactory result. The amino acids obtained from ester Fraction IV, which were not identified, weighed 3·2 g.

The distillation residue.

The esters remaining in aqueous solution after extracting phenylalanine ester with ether from the distillation residue were hydrolysed by boiling with baryta for several hours. The deposit which formed on long standing contained no aspartic acid. The cold filtrate was made neutral to litmus with sulphuric acid as a preliminary to the complete removal of barium. The precipitate which resulted was flocculent and had not the appearance of barium sulphate. It was filtered off, shaken with cold water, decanted several times, and washed many times on the filter with cold water. On investigation this precipitate was found to contain 3·5 g. amino acid material consisting of two unidentified portions, one of which was precipitated by phosphotungstic acid, and aspartic acid which was separated easily and of high purity.

The flocculent precipitate was extracted with successive quantities of boiling dilute sulphuric acid until the extract came through colourless. The solution was reduced in volume, and while at the boiling point sufficient baryta was added to reduce the content of sulphuric acid to 5 %. After heating on the water-bath and filtering, the fluid was cooled and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was complete. The precipitate was decomposed in the usual way, and yielded 0·64 g. material brown in colour, not hygroscopic, and strongly acid to litmus. The filtrate was freed from phosphotungstic and sulphuric acids, and evaporated to dryness on the water-bath. The dry material weighing 2·88 g. was extracted and washed with a small quantity of ice cold water, yielding a very soluble slightly coloured portion, and a perfectly white insoluble part, weighing 0·85 g. which proved to be aspartic acid. It was found to contain 36·13 % C and 5·27 % H; calculated for aspartic acid, 36·09 % C and 5·26 % H.

A portion was converted into the copper salt, which separated from a dilute aqueous solution in the characteristic form.

The fraction soluble in the ice cold water was converted into the copper

salt, which would not crystallise from water. It dried to a dark blue brittle material, pale blue when powdered, and insoluble in alcohol. Its identity was not established. The analytical figures, given below, are not unlike those obtained for some of the copper salt fractions described on page 389.

Copper	25·86 %
Nitrogen ...	8·0
Carbon	33·14
Hydrogen ...	4·14
Water lost at 125°	3·84

From the foregoing it appears that considerable losses of aspartic acid may occur when barium is removed quantitatively as the sulphate from amino acid solutions containing this substance. In all probability the considerable amount of nitrogen rigidly retained by the large barium sulphate precipitate removed in the early stages of the previous sulphuric acid hydrolysis (p. 379) is partially due to aspartic acid.

The solution from which the flocculent barium precipitate had been removed was made to contain 5 % sulphuric acid, and 25 % phosphotungstic acid dissolved in 5 % sulphuric acid added until the supernatant fluid had lost its brownish colour. This precipitate was removed, and a second fraction obtained by continuing the treatment with phosphotungstic acid until the precipitation was complete. These precipitates were decomposed in the ordinary way. The first one yielded 9·18 g. brown material acid in reaction to litmus. The second gave 10·1 g. amino acids alkaline to litmus from which 3·31 g. lysine picrate was obtained by adding an alcoholic solution of picric acid.

The amino acids not precipitated by phosphotungstic acid. On working up the filtrate from the phosphotungstic acid precipitates, a syrup weighing 24·7 g. very acid to litmus was obtained. This was extracted with alcohol. The insoluble part yielded nothing crystalline from water.

A concentrated aqueous solution of the extract gave two small successive fractions on long standing in a desiccator over sulphuric acid. The first, nodular in form, weighed 0·49 g., contained 62·54 % C, 6·93 % H, and 8·74 % N. Calculated for phenylalanine: 65·5 % C, 6·66 % H, and 8·48 % N. The odour of phenylacetaldehyde developed on warming a portion with dilute sulphuric acid and potassium bichromate.

In order to obtain dry matter suitable for analysis so that a general idea of the composition could be formed, the syrupy materials and their fractions were converted into copper salts, and the copper salts fractionated as described below.

Syrupy material insoluble in alcohol:

A. Copper salt crystallised from water.

B. Copper salt precipitated from concentrated aqueous solution by alcohol.

C. Copper salt, derived from material more soluble in water than that which gave copper salts A and B, precipitated from strong aqueous solution by alcohol.

Syrupy material dissolved by alcohol:

D. Copper salt, made from material which had crystallised from water as already described, precipitated by alcohol out of strong aqueous solution.

E. Copper salt precipitated from strong aqueous solution by alcohol.

F. Copper salt soluble in alcohol, precipitated by ether from alcoholic solution.

The analytical data for these copper salts are given in the following table:

	A	B	C	D	E	F
Carbon	—	32·90	31·54	—	35·15	39·79
Hydrogen	—	4·90	4·88	—	4·64	5·35
Nitrogen	8·0	8·51	8·80	—	8·0	8·83
Copper	26·11	26·18	28·74	27·57	23·63	18·90
Amino nitrogen	—	—	6·84	—	3·45	1·34

These copper salts swelled up considerably during ignition, and gave very disagreeable smells. The first four were blue when moist, and light blue in colour when dry. E gave a deep blue water solution, and a pale greenish blue dry salt precipitated by alcohol. F gave the deepest possible blue alcoholic solution, and a very pale blue dry salt when precipitated by ether.

Copper salt B. The copper was removed from this salt, and the aqueous solution concentrated to the syrupy stage. A very small quantity of nodular crystals separated on long standing, which were removed and recrystallised. The dried material weighed 0·18 g., softened at 180°, completely melted at 215°, gave a strong pyrrole reaction when warmed with zinc dust, and contained 44·42 % C, 6·78 % H, and 11·08 % N. These figures correspond approximately to a calculated formula of $C_5H_9O_3N$. The specimen was too small for conclusive identification.

Copper salt C. The copper was removed and 0·0493 g. crystals resembling glutamic acid were obtained from the concentrated aqueous solution. M.P. 208°. Contained 9·58 % N, calculated for glutamic acid 9·52 % N.

Copper salt E. Gave the pyrrole reaction on heating with zinc dust, but not so strongly as copper salt F.

Copper salt F. Weight = 2·5 g. Strong pyrrole reaction. Approximate formula for the free substance calculated from the analytical figures was $C_5H_9O_3N$. Impurity represented by the amino nitrogen (15·8 % of the total N) probably present. The properties and conditions strongly favour the view that the main constituent was the copper salt of pyrrolidonecarboxylic acid, the copper content differing by only 1 % from theory.

The syrupy material. Discussion.

No way was found for isolating a chemical individual from this viscous matter. Salt F was probably the impure compound of pyrrolidonecarboxylic acid. The ratio N : O in salt E was practically 1 : 3·56, and about 1 : 3 in B and C. A trace of glutamic acid was isolated from C. The composition of the free acids calculated from the data given for the copper salts resembles that of the "gummy substance" obtained by the lime-alcohol method for dibasic

acids [Foreman, 1914]. There can be no doubt that the same material is represented. The total weight of unidentified matter obtained from the distillation residue, omitting diamino acids, was 43·7 g. A considerable quantity, probably of peptide nature, was acid to litmus although precipitated by the ordinary phosphotungstic method for separating diamino acids.

The unesterified residue.

The baryta residue obtained when the second lot of esters were liberated from their hydrochlorides, completely freed from esters by washing with chloroform, was suspended in water, and the baryta all neutralised with sulphuric acid at 40–50°. After heating on the water-bath, the barium sulphate was removed and washed. The liquid was reduced in volume, sulphuric acid added until 5 % was present, and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was completed. The precipitate decomposed in the usual way yielded 12·96 g. dry matter. The filtrate was freed from phosphotungstic and sulphuric acids, and as much of the free hydrochloric acid as possible removed on the water-bath. The syrup which resulted was taken up in water, made up to 200 cc., and by estimating in aliquot portions, the whole solution was found to contain 1·568 g. total N, 1·043 g. amino N (van Slyke), and 0·042 g. N as NH_3. The hydrochloric acid remaining was quantitatively removed and the liquid again treated with phosphotungstic acid as before. A small gummy precipitate was obtained, and the whole of the colour disappeared from the solution. The reagents were completely removed, the liquid evaporated, and made up to 200 cc. The amino acid content was estimated by evaporating an aliquot and drying the residue to constant weight at 100°; 10 cc. gave 0·503 g. Allowing for the portions removed in the nitrogen estimations, the unesterified residue therefore contained only 10·06 g. amino acids not precipitated by phosphotungstic acid.

The 0·5 g. obtained from the aliquot was returned to the main solution, and the volume reduced to about 50 cc. on the water-bath. 2·76 g. tyrosine crystallised.

Serine. The filtrate from the tyrosine was evaporated down to 10–15 cc. On standing over night, crystals in the form of thin hard transparent plates separated. This substance was recrystallised from water after removing a trace of colour from the solution by animal charcoal, and yielded crystals of the same character, which weighed 0·998 g. M.P. 231°, with decomposition. Analysis gave the following results:

	Found	Calculated for serine
	%	%
C	34·49	34·29
H	6·87	6·67
N	13·48	13·33

A syrup obtained from the remaining material. An attempt was made to obtain a second crop of serine, but when the concentration was carried further than before, the solution became viscous, and although crystals nodular in form appeared on standing, they could not be separated by suction. A very small quantity from which most of the liquid had been removed by suction gave a copper salt, deep blue in colour, insoluble in alcohol, which contained 19·56 % Cu. The viscous matter resembled cheese in consistency after standing a long time *in vacuo* over sulphuric acid. After treatment with a little cold water, a few large hard crystals were picked out, washed, and recrystallised. This specimen weighed only 0·2 g., and melted at 213°.

Material separated from the syrup by means of basic lead acetate and alcohol. A satisfactory precipitate was obtained by means of alcohol after adding basic lead acetate solution to the aqueous solution of the syrup. The addition of alcohol was continued until the separation was complete. The lead was removed from the washed precipitate, and the resulting solution reduced to very small volume. Large white spherical nodules separated from the viscous solution on standing over night. These were removed, and a second fraction of the same type obtained in the same way. The fractions were recrystallised from water.—No. 1 gave 0·123 g., M.P. 182°; No. 2, 0·05 g., M.P. 181°. A trace heated with zinc dust gave a very strong pyrrole reaction. The material, which appeared to be definite in character, gave the following results on analysis:

C	39·97 %
H	6·33
Amino N	9·94

Although the percentages do not differ widely from those of glutamic acid, further work showed that the substance would not give a hydrochloride insoluble in saturated aqueous hydrochloric acid. The total weight of material precipitated by basic lead acetate and alcohol was 2·23 g.

The amino acids not precipitated as lead compounds. The filtrate from the lead precipitate was freed from lead. Spherical nodules separated from the concentrated aqueous solution on standing. The mother liquor on further concentration yielded crystals of the same kind; M.P. 263° after recrystallisation from water. These gave a strong pyrrole reaction. The quantity obtained was too small for analysis. (Hydroxyproline melts at 270°.)

Discussion.

The unesterified residue contained only 10·06 g. monamino acids. The identifiable part consisted of tyrosine and serine, which are both hydroxy acids. The remainder was syrupy. No traces of known amino acids containing two oxygen atoms in the molecule were found. Further work may show that only the lead salts of hydroxy acids resist esterification. The two small specimens derived from the lead-alcohol precipitate seemed to be definite in character, and the analytical data are not inconsistent with the view held that the substance was probably a hydroxy acid.

The yield of monamino acids.

The amount of each monamino acid separated in the course of the foregoing investigations will be found in the following table:

Amino acid	Reference page	Weight in g.		%
Glycine	385	1·24		
,,	385	0·04	1·28	0·45
Alanine	385		5·34	1·85
Valine	386		22·88	7·93
Leucines	386		27·97	9·70
Proline	386		22·01	7·63
Phenylalanine	384	10·70		
,,	388	0·49	11·19	3·88
Serine	390		1·0	0·35
Aspartic acid	387	0·48		
,,	387	0·51		
,,	387	0·85	1·84	0·64
Glutamic acid	381		45·87	15·91

Discussion.

With the exception of aspartic acid and serine, the percentages are all appreciably higher than those given by Osborne and Guest [1911] in their compounded table which shows the highest reliable figures for each amino acid obtained from caseinogen up to that date. The yield of aspartic acid is much lower than that obtained in Hydrolysis *A* (p. 379), owing to the fact that no liquid air was available when the esters were distilled.

The new method for preparing esters.

The older methods for preparing the esters of the amino acids leave much to be desired from the point of view of yield. This is chiefly due to the fact that water is added to the ester hydrochlorides and the esters are subjected to the saponifying influence of aqueous alkali during their liberation and extraction. Levene and van Slyke [1909] brought about a considerable reduction in the content of free alkali in the aqueous solution by substituting anhydrous baryta for the soda or potash previously employed and better yields resulted. The esters however are exposed, in their method, to aqueous baryta during the extraction which occupies three-quarters of an hour, and a certain amount of saponification occurs. Their original syrup contains a considerable amount of free hydrochloric acid as well as the ester hydrochlorides, and on adding the baryta a large amount of heat of neutralisation as well as further water develop within a thick mass which in a few minutes becomes semi-liquid and alkaline. In spite of the cooling by means of a freezing mixture, local overheating in such material appears likely. The mass eventually breaks up into granules, remaining in this condition until the operations are concluded. A complete extraction of such material, for the greater part of the time in a wet condition, by stirring with successive additions of ether, seems highly improbable. Saponification and incomplete extraction probably account for the

results obtained by the authors, expressed by them as follows: "Extraction as described...yields a second smaller crop of esters, and by repeating the process a third and even a fourth crop in decreasing yields may be obtained."

Esterification and saponification are both influenced in degree by the amount of water present. The new method for the preparation of esters was designed to eliminate the adverse effect of water upon the yield. As the extraction of esters from large bulks of moist material with ether is probably very incomplete, the esters are liberated in a large volume of a more suitable solvent which protects them from saponification, and from which the residue can be filtered and washed. The following statements will explain the procedures adopted:

(1) The water of the original solution of amino acids is eliminated by obtaining the amino acids in the form of dry lead salts.

(2) In the esterification the reverse reaction is prevented by a large excess of alcohol and the favourable influence of the lead chloride formed.

(3) Excess of free hydrochloric acid is removed from the ester hydrochloride solution without the formation of water.

(4) In the absence of free hydrochloric acid, the water of esterification can be removed with the alcohol from the alcoholic solution of the ester hydrochlorides.

(5) The ester hydrochlorides readily dissolve in chloroform, which can be very easily dehydrated by means of anhydrous baryta.

(6) The amount of water produced by the action of the baryta upon the hydrochloric acid part of the ester hydrochloride molecules is small, as two molecules of hydrochloride are required to give one of water. Water is very sparingly soluble in chloroform (100 cc. dissolve 0·152 cc. water at 22° [Herz, 1898]), and the mass of anhydrous baryta relative to that of the water produced is very large. The water is therefore rapidly taken up. The esters on the other hand are very readily soluble in chloroform, and consequently are removed from the spheres of action immediately. There is practically no exposure of the esters to the influence of aqueous baryta, and saponification is prevented. The increase in temperature which occurs produces little if any ill effect upon the yield, and cooling by means of freezing mixtures is unnecessary. The esters are completely separated from the residue by the simple method of filtering and washing.

The efficiency of the method. The second crop of esters obtained in Hydrolysis *B* weighed only 25·6 g. and probably included a little ether. The opinion was formed that this quantity consisted of the esters of dibasic material, and was practically free from the monobasic acids whose esters appear in the first three fractions of the ester distillation. It is regretted that this portion was not investigated separately. The opinion was based upon the following facts. In Hydrolysis *A*, the yield of glycine, alanine, valine and leucines, obtained from a single ester preparation, together amounted to 18·2 %, and the proline was 6·98 %. The corresponding figures for Hydro-

lysis *B*, in which the second crop of 25·6 g. esters was combined with the first before distillation, were 19·93 and 7·63 respectively. As yield was the chief object of Hydrolysis *B*, the experiments were more carefully conducted and the caseinogen was hydrolysed by means of hydrochloric acid, whereas in Hydrolysis *A* sulphuric acid was employed. The yields are generally not so good when sulphuric acid is used, probably because the hydrolysis is not carried so near to completion and loss of amino acids occurs on removing the large barium sulphate precipitate (p. 379).

In another hydrochloric acid hydrolysis of a kilo of "Hammersten casein," equal to 901·3 g. of the pure substance, one preparation of esters by the new method was made, and the first three ester fractions were distilled in the same manner and up to the same temperature as in Hydrolysis *B*. After extracting the amino acids of these three fractions with hot alcohol, 163·03 g. of a mixture of glycine, alanine, valine and leucines resulted, which corresponds to a total yield of 18·1 %. The small quantities of these amino acids which separate in the ordinary way from the proline extract before it will enter completely into solution in cold alcohol are not included in the calculation. The freedom of the unesterified residue of Hydrolysis *B* from amino acids of the leucine type supports the view that the small second crop of esters contained practically none of these substances.

The very large distillation residue obtained, and the very small quantity of monamino acids found in the unesterified residue, are facts which also point to the high efficiency of the new ester method.

The fractionation of the esters. In distilling the esters of Hydrolysis *B*, four fractions were obtained without the aid of liquid air. After Fraction III was complete, a rise of 6° "vapour" temperature and 20° in the "bath" occurred before further esters commenced to distil over. Fraction IV yielded only 1·36 g. phenylalanine hydrochloride, while nearly nine times as much was obtained quite as easily from the distillation residue. The other material derived from Fraction IV was apparently free from amino acids of the leucine type, and, so far as the estimations were concerned, no advantage was gained by distilling this fraction. Now that the dibasic acids can be easily and completely removed before esterification, their estimations do not depend upon the working up of the higher boiling esters, a method which usually gave very poor yields, owing to the large amount of decomposition. If serine is present in the distillation residue, it will probably crystallise quite readily in the absence of dibasic acids, syrupy products and phenylalanine. It appears, therefore, that there is no need to continue the ester distillation further than Fraction III if the work is carried out on the lines suggested, and the esters of the distillation residue will not be subjected to the influence of the higher temperatures.

Improvements suggested by the results of later experiments.

The foregoing experiments were completed in 1913. Further work showed that the syrupy material found in such large quantity in the distillation residue, as well as to some extent in the unesterified fraction, can be removed from the original hydrolytic mixture by the "lime-alcohol" method for separating dibasic amino acids [Foreman, 1914].

Further investigations on the nature of the new syrups prepared under certain temperature precautions, and on the possibilities of the "lime-alcohol" method for simplifying the mixture of amino acids before esterification, were carried out in the latter part of 1914. The work was suspended early in 1915 owing to war conditions. The experiments will be described in a further paper, and the results compared with those obtained by Dakin [1918] who has since identified a new amino acid, β-hydroxyglutamic acid, in the syrups which the "lime-alcohol" method enabled him to obtain.

The further investigations showed that on working up the alcoholic filtrate from the insoluble calcium salts, the monobasic amino acids can be obtained in the form of a light brown powder, proving that all the viscous matter can be removed by the "lime-alcohol" method. Impure proline was obtained by extracting the powder with alcohol. The ester process however will probably yield purer proline.

The advantages likely to be gained by removing the alcohol-insoluble calcium salts before applying the new method for preparing esters are given in the following summary:

(1) A single ester preparation will contain the whole of the esters of the monobasic monamino acids, with the possible exception of those of the hydroxy type.

(2) The esters will fractionate more easily, and the difference between "bath" and "vapour" temperatures will be reduced, thus decreasing the risk of decomposition.

(3) Substances of syrupy nature will not be present to complicate the issue at almost every stage. The separations will therefore be more complete, and the amino acids yielded in purer condition.

(4) The residues will be very much simpler in composition, and the amino acids they contain thrown into much greater relief.

Composition of caseinogen.

The sum of the percentages of the known amino acids separated from the products of caseinogen by the older methods showed a very considerable deficit. The method for estimating diamino acids gives very satisfactory results, and consequently it is generally believed that the loss falls almost entirely upon the monamino acids during their isolation and estimation. The preparations of esters by the older methods were incomplete, and definite information in regard to the nature of the amino acids making up the de-

ficiency could not be gained by examining the residues. The methods of preparing and distilling the esters of Hydrolysis *B* made a better examination of the residues possible. The distillation residue yielded material probably of peptide character (p. 390), indicating that a complete hydrolysis of caseinogen is not effected by boiling for 48 hours with hydrochloric acid, as well as a large amount of syrupy material of unknown constitution. The amount of monamino acids in the unesterified residue was very small and consisted of hydroxy acids together with further syrups. Neither of these residues gave any indication that amino acids of the leucine type were present. The syrupy material was afterwards separated directly from the hydrolytic products, together with glutamic and aspartic acids, in the form of alcohol-insoluble calcium salts [Foreman, 1914], and as much as 37·88 % of the caseinogen was accounted for by this method.

Our present knowledge of the quantitative proportions of the products obtained by the acid hydrolysis of caseinogen, including the results of these investigations, may be summarised as shown in the accompanying table. The percentage of lysine was calculated from the figures given by van Slyke [1913] for his estimation of this substance by the Kossel method. The figures for the other amino acids represent the highest reliable results as selected by Osborne and Guest [1911] in their review of the information then available. A total of 67·85 % of caseinogen was accounted for by them in this manner, including the figures for elementary sulphur and phosphorus.

Results of Hydrolysis *B* (1913)	Glycine	0·45 %
	Alanine	1·85
	Valine	7·93
	Leucines	9·7
	Proline	7·63
	Phenylalanine	3·88
Results obtained by the method for separating dibasic amino acids [Foreman, 1914]	Glutaminic acid	21·77
	Aspartic acid	1·77
	New Syrups	14·34
Van Slyke	Lysine	7·62
Results selected by Osborne and Guest ...	Histidine	2·5
	Arginine	3·81
	Tryptophan	1·5
	Serine	0·5
	Cystine	—
	Tyrosine	4·5
	Hydroxyproline	0·23
	Diaminotrihydroxydodecanic acid	0·75
	Ammonia	1·61
	Sulphur	0·76
	Phosphorus	0·85
		93·95
Obtained from Hydrolysis *B*, probably of peptide nature (p. 390)		3·41
		97·36

The products include the water of hydrolysis, and the total should be greater than 100. The application of the new ester method after removing the

dibasic material will very probably throw considerable further light upon the composition of caseinogen, and further investigation is proceeding on these lines.

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XXXVII. ON AMINO-ACIDS. PART II. HYDROXYGLUTAMIC ACID.

By HENRY DRYSDALE DAKIN.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

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The object of the following communication is to record the results of experiments made in continuation of work already published in this *Journal* [Dakin, 1918]. In the previous communication it was shown that the products of hydrolysis of a protein, *e.g.* caseinogen, might be extracted with partially miscible solvents, such as butyl alcohol, so that the feebly ionised monamino-acids and proline were substantially removed[1], while the strong acids and bases remained behind. Evidence was presented of the occurrence among the acids, in addition to glutamic and aspartic acids, of a new amino-acid which was regarded as β-hydroxyglutamic acid from a study of its composition and reactions. The present paper contains the results of the further study of this acid together with experiments on its synthesis and that of allied substances. For convenience, the subject-matter is divided into the following sections:

I. On the synthesis of inactive β-hydroxyglutamic acid and allied substances.

II. The *p*-nitrophenylosazones of malic semi-aldehyde and tartronic semi-aldehyde.

III. Optical activity of natural β-hydroxyglutamic acid.

IV. Alkaloid salts of β-hydroxyglutamic acid and other amino-acids.

V. Identification of β-hydroxyglutamic acid among the products of hydrolysis of glutenin and gliadin.

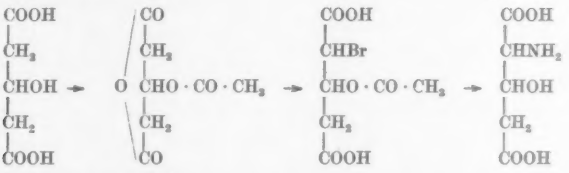
VI. The fate of β-hydroxyglutamic acid in the diabetic organism.

I. On the synthesis of inactive β-hydroxyglutamic acid and allied substances.

At first sight it appeared that the synthesis of inactive β-hydroxyglutamic acid would be a relatively simple problem capable of solution by many methods,

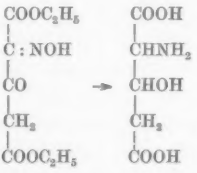
[1] It is perhaps worth recording the fact that if the amino-acid aqueous solution is saturated with potassium carbonate and then extracted with ordinary ethyl alcohol, the monamino-acids are found in the alcoholic extract as potassium carbamates and may be precipitated by acetic acid.

but, at any rate in the writer's experience, this has not proved to be the case. The first method that was tried consisted in brominating the acetyl derivative of β-hydroxyglutaric anhydride described by Blaise [1903] and then acting on the product with ammonia:



The acetyl derivative is obtained by the action of acetic anhydride on β-hydroxyglutaric acid, prepared by the reduction of acetonedicarboxylic acid. But while the bromination apparently went smoothly, the action of ammonia removed the halogen without introducing an amino-group, probably through the formation of a trimethylene derivative. An analogous reaction was observed by Perkin and Tattersall [1905] in the case of α-bromoglutaric acid.

A more promising method presented itself in the reduction of α-isonitroso-acetonedicarboxylic ester:

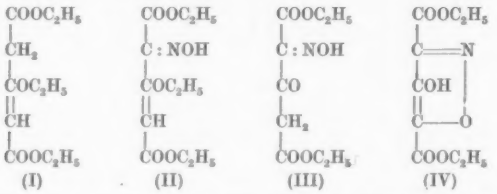


A very large number of experiments were made with this substance, using almost every known reducing agent that had the smallest prospect of success, but the reaction did not proceed smoothly. Using sodium or sodium amalgam in neutral or acid solution, it was an easy matter to reduce the ester and to obtain an acid which, in many ways, resembled the natural hydroxyglutamic acid and had approximately the same composition. But a variable proportion of the product when tested with nitrous acid was found not to be an amino-acid nor was it a hydroxypyrrolidonecarboxylic acid for it did not give an amino-acid on warming with hydrochloric acid. The separation of the products of reaction proved very difficult and has not been satisfactorily accomplished. It appeared that complicated condensations followed the production, as the first stage of the reaction, of α-amino-acetonedicarboxylic ester, such as are known to occur with amino-acetoacetic ester. It therefore seemed desirable to limit the reactivity of the carbonyl group. The introduction of an acetyl group attached to oxygen, by means of acetyl chloride in pyridine solution, proved of no avail. Later the observation was made that ethoxyglutaconic ester, obtained by the action of orthoformic ester on acetonedicarboxylic ester, on reduction with sodium amalgam in the presence of excess of carbon dioxide

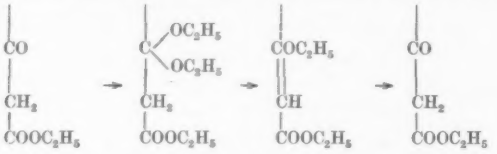
gave β-hydroxyglutaric acid instead of β-ethoxyglutaric acid as might be expected:

$$COOEt \cdot CH_2 \cdot CO \cdot CH_2 \cdot COOEt \rightarrow COOEt \cdot CH{=}COEt \cdot CH_2 \cdot COOEt \rightarrow COOH \cdot CH_2 \cdot CHOH \cdot CH_2 \cdot COOH$$

It therefore appeared possible that α-isonitroso-acetonedicarboxylic ester might be converted into isonitroso-β-ethoxyglutaconic ester (II) and that the latter might be successfully reduced to hydroxyglutamic acid. Orthoformic ester was found to react vigorously with isonitroso-acetonedicarboxylic ester but the product, which cannot be distilled, sometimes gave results on analysis indicative of the ethoxy ester, but often, after simple manipulations and careful drying, nothing but unchanged nitroso-acetonedicarboxylic ester was obtained. It was clear that the ethoxy group was very easily removed and indeed on reversing the order of the reactions and starting with ethoxyglutaconic ester (I) it was found that nitrous acid could convert it into nitroso-acetonedicarboxylic ester (III) and when present in excess into hydroxyisoxazoledicarboxylic ethyl ester (IV):



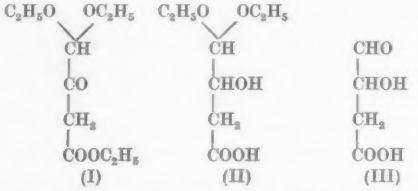
As is well known from Claisen's researches, the initial reaction between a β-ketonic ester and orthoformic ester in the presence of a suitable catalyst involves the formation of a γ-diethoxy ester which usually parts with a molecule of alcohol to give an ethoxy derivative of an olefinic acid, but which in turn may be reconverted into the original ketonic ester by simple hydrolysis:



Accordingly the reaction between orthoformic ester and isonitroso-acetonedicarboxylic ester was effected at low temperatures, and in order to limit decomposition of the product, it was reduced with sodium amalgam after a minimum of manipulation. The results were rather more satisfactory than those obtained by the direct reduction of isonitroso-acetonedicarboxylic ester, and small amounts of an amino-acid were eventually isolated which bore a very close resemblance to natural β-hydroxyglutamic acid. However, the final stage in the preceding synthesis does not proceed as smoothly as might be desired, and as the amino-acid to be finally isolated resembles the natural acid in being extremely soluble and difficult to crystallise or characterise by

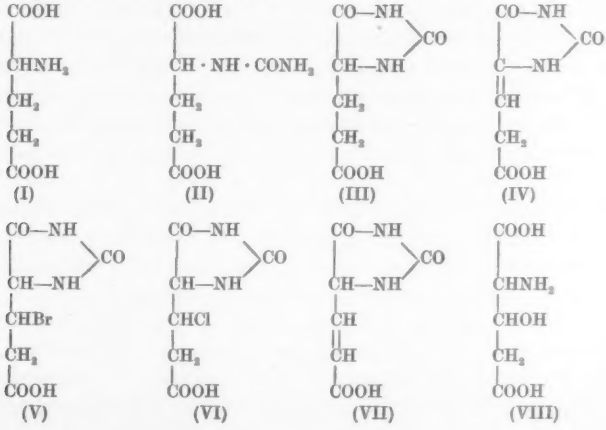
derivatives, and, moreover, readily passes over into a pyrrolidone derivative with loss of water, it appeared desirable to attempt some other mode of synthesis, as a confirmation.

The next method to be tried was based upon Strecker's well-known synthesis of amino-acids from aldehydes by way of the amino-nitriles. For this purpose it was necessary to prepare the hitherto unknown semi-aldehyde of malic acid, *i.e.* β-hydroxy-γ-aldehydobutyric acid. This was successfully accomplished in the following way—γ-diethoxyacetoacetic ester (I) obtained as previously described by Dudley and the writer [1914], was reduced with sodium amalgam to γ-diethoxy-β-hydroxybutyric acid (II). The latter substance, on careful hydrolysis with dilute sulphuric acid, gave malic semi-aldehyde (III):



Unfortunately, the malic semi-aldehyde, when subjected to Strecker's reaction substantially under the same conditions as those used by Fischer in the synthesis of serine from glycollic aldehyde, gave only a negligible trace of an amino-acid. From 20 g. of the aldehyde, only enough amino-acid was obtained to observe its qualitative reactions, which resembled those of the natural acid, and to furnish about 0·2 g. of its silver salt. Attempts to improve the yield were unavailing and the method, which seems often to prove unsatisfactory in the case of hydroxyaldehydes, was abandoned.

More satisfactory results were obtained with a synthesis starting with glutamic acid. Glutamic acid (I) was converted in α-uraminoglutaric acid (II)



by the action of potassium cyanate, and hydantoin-propionic acid (III) was obtained from the latter by warming with hydrochloric acid, as already described [Dakin, 1910]. The action of bromine on hydantoin-propionic acid was then studied. It was found that on warming hydantoin-propionic acid dissolved in excess of glacial acetic acid with a molecular proportion of bromine, the latter quickly disappeared and that much more than half of the bromine was obtained as hydrobromic acid. The products of the reaction reduced permanganate solution freely and could absorb further amounts of bromine, so that it was clear that unsaturated products had been formed. On brominating concentrated solutions in glacial acetic acid, it was found that less of the bromine appeared as hydrobromic acid and a crystalline mixture of substances was separated which appeared to be made up of the unsaturated acid together with its hydrobromic acid addition product. The latter could be readily separated in the form of colourless cubes by repeated crystallisation from hydrobromic acid solution and from its reactions and composition is regarded as hydantoin-β-bromopropionic acid (V). It is reduced by zinc dust to hydantoin-propionic acid and, like most β-bromo-compounds of glutaric acid, is very unstable towards alkali. The unsaturated acid was obtained by boiling the mixture just referred to with zinc dust, separating it as zinc salt and then decomposing the latter with hydrogen sulphide. It is a sulphur yellow substance, reducing permanganate freely, and is convertible into hydantoin-β-bromopropionic acid with hydrobromic acid. It absorbs bromine slowly in the cold when dissolved in acetic acid but rapidly at 100°. This substance, to which is assigned the formula (IV), is somewhat difficult to name satisfactorily but may be regarded as *hydantoin-β . γ-propenylic acid*. Its relation to glutaconic acid is clear and the possibility of *cis* and *trans* isomers is also evident. It is not improbable that it is formed by the removal of hydrobromic acid from a bromohydantoin-propionic acid in which the bromine is in the γ-position.

Additional evidence as to the constitution of hydantoin-β-bromopropionic acid and its formation by the addition of hydrobromic acid to hydantoin-β . γ-propenylic acid is furnished by the following facts. In the first place, the yield of this substance is increased by brominating hydantoin-propionic acid in the presence of excess of hydrobromic acid. Secondly, although the hydantoin-propionic acid is strongly optically active, the β-bromo-derivative is entirely inactive although containing an asymmetric carbon atom. This result is readily interpreted if the reaction takes place as indicated, for the intermediate unsaturated acid (IV) has lost its asymmetry. Lastly, if the bromination of hydantoin-propionic acid is carried on in fuming hydrochloric acid solution, hydantoin-β-chloropropionic acid (VI) is readily obtained.

A close analogy to the first stage of the reaction under discussion is furnished by the action of bromine upon p-hydroxybenzylhydantoin which was found by Johnson and Hoffman [1912] to give the unsaturated 3·5-dibromo-4-hydroxybenzalhydantoin. Gabriel [1906] also observed a similar

change in the formation of "pyvureidic acid" on brominating hydantoin-acetic acid.

The conversion of hydantoin-β-bromopropionic acid into β-hydroxyglutamic acid, a reaction involving the opening of the hydantoin ring by hydrolysis and replacement of the halogen by hydroxyl, is not as easy as might be wished. On warming the acid with excess of barium hydroxide, a clear solution is at first obtained, but in a few moments an insoluble barium salt separates out. This salt is a mixture which contains progressively less nitrogen as the amount of barium hydroxide and time of heating are increased and is mainly made up of barium salts of acids containing two or possibly three carbon atoms together with barium carbonate. On prolonged boiling with excess of barium hydroxide traces of an amino-acid are formed, but the bulk of the substance undergoes complete decomposition. On boiling with pyridine or other bases, the halogen of hydantoin-β-bromopropionic acid is readily removed, but carbon dioxide is evolved and neutral products result. The instability of the five carbon chain of hydantoin-β-bromopropionic acid towards alkali is not surprising in view of the ease with which β-bromoglutaric acid is known to give vinylacetic acid when warmed with soda [Wislicenus, 1899; Ssemenov, 1899].

Better results were obtained on simple boiling of hydantoin-β-bromopropionic acid with water. Hydrobromic acid is slowly split off, and among other complex products a crystalline substance is easily separated in about 20 % yield, which is an unsaturated acid, isomeric with the hydantoin-$\beta.\gamma$-propenylic acid already described. Like the latter substance, it is a bright sulphur yellow compound and absorbs hydrobromic acid and bromine on warming. This substance appears to be hydantoinacrylic acid as represented by formula (VII). On prolonged treatment of this substance with hot barium hydroxide solution, the hydantoin ring is opened with removal of ammonia and carbon dioxide, while the elements of water are taken up with formation of β-hydroxyglutamic acid. The opening of the hydantoin ring by alkaline hydrolysis is of course a common reaction, as is the formation of a β-hydroxy-acid from an unsaturated acid by treatment with alkali, *e.g.* glutaconic acid gives β-hydroxyglutaric acid and fumaric acid yields malic acid. The yield of amino-acid is disappointing, amounting to about 20 % of the hydantoinacrylic acid taken. Calculated on the glutamic acid used as the starting material, the yield is not more than about 2 %. An alternative method of converting hydantoin-β-bromopropionic acid into hydroxyglutamic acid consisted in hydrolysing the former with fuming hydrochloric or hydrobromic acid in a sealed tube at 130°, and subsequently removing the excess of acid and boiling the product with lime water to replace the halogen by hydroxyl. The results were not much more satisfactory than those just described.

The synthetic inactive β-hydroxyglutamic acid isolated as described in the experimental portion of the paper could not be obtained in large enough amounts to permit of a detailed study, but the following points of resemblance

to the natural optically active acid were noted and no dissimilarities were observed.

The acid is extremely soluble in water and crystallises with great difficulty. It is insoluble in alcohol and most organic solvents. The whole of its nitrogen is in the NH_2 form and can be liberated by nitrous acid in van Slyke's apparatus. On heating to 105°, almost the whole of the nitrogen goes over into the (NH) form, through formation of a hydroxypyrrolidonecarboxylic acid with loss of water, and no longer reacts with nitrous acid. The natural acid shows precisely the same behaviour and the ease with which it parts with water accounts for the lack of a sharp melting point. The copper salt, like that of the natural acid, is very soluble in water, insoluble in alcohol. The acid titrates to litmus as a monobasic acid and forms neutral salts with the alkali metals containing one equivalent of base and alkaline salts containing two equivalents. The *silver salt* is a white granular precipitate almost insoluble in water, contains two equivalents of silver and resembles the salt of the natural acid closely. The acid is saturated and does not promptly reduce permanganate in the cold in alkaline solution.

On treating a few milligrams of the substance with diazobenzenesulphonic acid and then adding sodium hydroxide, a cherry red colour develops (or orange red if the solution is very dilute) which becomes intensely deep on warming[1]. If sodium carbonate be used as alkali, the colour tone in the cold is orange red which deepens to cherry red on warming. The natural acid, separated from its crystalline strychnine salt, shows the same reaction. Lastly, on gently warming a few milligrams of the acid dissolved in a drop or two of water with β-naphthol and concentrated sulphuric acid, a brilliantly fluorescent yellowish green solution results[2]. The same reaction is shown by the natural acid and by malic acid.

Since β-hydroxyglutamic acid contains two dissimilar asymmetric carbon atoms it may occur in four active forms and at least two inactive forms. It has not been possible to obtain sufficient of the inactive synthetic acid for resolution into active forms so that the space relationships of both active and synthetic acid await further investigation.

In order to save unnecessary duplication by other workers, it may be of some value to indicate a number of other attempted syntheses of β-hydroxyglutamic acid which were tried without success, though of course it is quite

[1] Most amino-acids on *boiling* with diazobenzenesulphonic acid and caustic soda develop a reddish colour, but the reaction is trivial compared with the above. Rosenthaler [1912] has described an analogous reaction for malic acid.

[2] In the original description of the natural acid it was stated that it gave a violet red colour with resorcinol and sulphuric acid. This is an error and is apparently connected with the use of silver nitrate in separating the acid. Nitric acid gives a similar reaction, but preparations of the amino-acid containing so little nitric acid that no reaction could be obtained with the brucine test still gave a positive reaction. When no silver nitrate was used in the separation of the acid, the characteristic reaction with resorcinol was not obtained. Possibly it is due to a trace of some oxidation product.

possible that some other of them might be successful if more suitable conditions were found than those employed by the writer:

(*a*) Bromination of benzoylglutamic acid, with the hope of obtaining a benzoylamino-glutaconic acid by removal of hydrobromic acid.

(*b*) Reduction of *o*-toluidine-azo-acetonedicarboxylic ethyl ester [cp. Bülow and Göller, 1911].

(*c*) Reduction of various hydrazine derivatives of acetonedicarboxylic ester.

(*d*) Condensation of oxymethylenehippuric ester with malonic ester.

(*e*) Condensation of dichloracetal with malonic ester followed by reactions analogous to Leuch's synthesis of serine.

(*f*) Reduction of isonitrosoglutaconic ester.

(*g*) Reduction of hydroxyisoxazoledicarboxylic ethyl ester.

The isolation of β-hydroxyglutamic acid from the products of hydrolysis of proteins makes it desirable to know more of related hydroxy-amino-dicarboxylic acids. Reference was made in a previous paper [Dakin, 1918] to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. It was there stated that "As Fischer has pointed out Skraup's experiments are not described in a form capable of repetition and no evidence other than the analysis of a copper salt is adduced to show that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon or hydrogen determinations were made on the free acid." Almost immediately after Skraup's announcement, Neuberg stated that he had obtained hydroxy-aspartic acid in small amount by the action of nitrous acid on diaminosuccinic acid. Erlenmeyer and Bade [1904] state that they synthesised the same substance by reducing oxalylhippuric ester, but no account of the process or product has yet appeared, so far as we have been able to ascertain. The writer has endeavoured to synthesise hydroxyaspartic acid and has found that it is formed by the action of ammonia on barium chloromalate, a reaction which is similar to one partially investigated by Lossen. A detailed account of the acid, which has entirely different properties from those described by Skraup and by Neuberg, will form the subject of a separate communication.

It is a curious fact that the amino-acid gives a so-called abnormal copper salt, resembling iso-serine in this respect, as was shown by Fischer. The presence of a hydroxyl group contiguous to a carboxyl group is undoubtedly responsible for the fact that the copper salt contained three equivalents of copper instead of two. The structural similarity between iso-serine which combines with two equivalents of copper and hydroxyaspartic acid is clear from the following formulae:

$CH_2(NH_2) \cdot CHOH \cdot COOH$	Iso-serine,
$HOOC \cdot CH_2(NH_2) \cdot CHOH \cdot COOH$	Hydroxyaspartic acid.

The formation of this abnormal copper salt of synthetic hydroxyaspartic acid is clearly of value in further questioning the identity of Skraup's acid which was considered to be a hydroxyaspartic acid largely on the basis of the analysis of a copper salt which was believed to possess a normal composition.

Experimental.

In the following section, the experiments leading to the synthesis of β-hydroxyglutamic acid from hydantoin-propionic acid will be considered first, since they have given the most satisfactory results. The preparation of malic semi-aldehyde and its derivatives will then be described. Only a brief outline of experiments on the reduction of isonitroso-acetonedicarboxylic ester and its condensation products with orthoformic ester will be included since they cannot be regarded as satisfactorily completed. The melting points recorded are all "uncorrected" observations, unless otherwise indicated.

Hydantoin-propionic acid. The preparation and properties of this substance have already been described by the writer [1910]. Certain minor changes in its preparation have proved advantageous as regards the yield. Glutamic acid, 29·4 g., is heated on the water-bath with about 100 cc. of water, and strong sodium hydroxide solution is gradually added until the whole of the acid is dissolved and the reaction of the solution is approximately neutral to litmus. To the solution of mono-sodium glutamate thus obtained 20 g. of freshly prepared potassium cyanate (1·2 mols.) is added and the mixture allowed to evaporate slowly over the water-bath for about an hour. The solution is then made acid to Congo-red with hydrochloric acid and an additional 30 cc. of the concentrated acid is added. The solution is then heated in a covered dish on the water-bath for two or three hours in order to convert the uramino-acid first formed in the alkaline solution into hydantoin-propionic acid. The mixture is next transferred to a capacious flask and evaporated to dryness under diminished pressure. The residue is then extracted with boiling alcohol, thus dissolving the acid and leaving sodium and potassium chlorides undissolved. The alcoholic solution is at once diluted with water and the alcohol removed by evaporation. Hydantoin-propionic acid slowly crystallises out of the concentrated solution in a yield fully equal to the weight of glutamic acid taken. The direct evaporation of the alcoholic solution without dilution with water should be avoided, as in this case an uncrystallisable syrup frequently results, possibly owing to ester formation.

Bromination of hydantoin-propionic acid. The first point to be investigated concerned the proportion of hydrobromic acid formed on acting upon hydantoin-propionic acid (1 mol.) with bromine (1 mol.) in glacial acetic acid solution. The experiments were conducted in small sealed tubes which, after heating at 90° until all the bromine had disappeared, were opened under water, acidified with nitric acid and the hydrobromic acid estimated volumetrically with standard silver nitrate solution. Appropriate blanks were carried out and the necessary corrections made. In experiments employing

0·2 g. of the acid and an equivalent amount of bromine in 1 cc. of glacial acetic acid, it was found that hydrobromic acid was liberated equivalent to from 1·5–1·78 molecular proportions. Slightly higher results were obtained on increasing the dilution with acetic acid and lower results if the solvent were reduced. It was therefore clear that a large proportion of any bromine entering the hydantoin-propionic acid molecule was removed as hydrobromic acid, but, as was subsequently found, the unsaturated products formed can again take up hydrobromic acid under suitable conditions.

On brominating larger quantities of the hydantoin-propionic acid dissolved in six parts of glacial acetic acid, as above described, and then mixing the resulting solution with water and crystallising after evaporation, a light yellow substance was obtained melting indefinitely around 210–220°. Under the microscope it was clearly seen to be a mixture and contained about 35 % carbon, 3 % hydrogen and about 20 % of bromine. This mixture furnished hydantoin-β-bromopropionic acid on repeated crystallisation from hydrobromic acid solution, while on reduction with zinc dust it gave hydantoin-$\beta.\gamma$-propenylic acid, as described later. The yield of crystalline mixture obtained amounted to about half the weight of acid brominated.

Hydantoin-β-bromopropionic acid. The preceding experiments clearly indicated that the bromine derivative of hydantoin-propionic acid was almost surely formed by the secondary addition of hydrobromic acid to an unsaturated intermediate product (hydantoin-$\beta.\gamma$-propenylic acid). It was therefore probable that it could best be prepared in a medium already containing preformed hydrobromic acid. Five parts of aqueous hydrobromic acid (50 %) were tried with satisfactory results, but the best yields were obtained as follows.

Hydantoin-propionic acid, 17·2 g., is boiled with 30 cc. of glacial acetic acid in a strong flask and while the mixture is still fairly hot, 35 cc. of glacial acetic acid previously saturated with hydrobromic acid at 0° are added and the mixture at once cooled under the tap. The acid will now readily dissolve and remain in solution. Bromine, 16 g., is added and the flask is then closed and gently warmed at about 60°–70° on a water-bath. In the course of an hour or two, the whole of the bromine will disappear and the mixture is then allowed to stand in a cool place for a day or two. After a time, a crop of white crystals separates and is removed by filtration, using an asbestos pad on a porcelain funnel. On concentrating the mother liquor under reduced pressure, further crops may be obtained leaving a thick syrup as mother liquor. The crystals are recrystallised from boiling 30 % hydrobromic acid and separate as perfectly white cubes and rhombic prisms melting at 228–230° with effervescence and turning deep brown in colour. If crystallised from water, slight decomposition takes place, giving a yellowish product. The yield amounts to 9–11 g. The substance reacts as a monobasic acid to litmus. On titrating 0·25 g. with $N/10$ sodium hydroxide 10 cc. were required, equivalent to a molecular weight of 250, compared with a calculated value of 251.

Analysis. 0·1253 g.; 0·1334 CO_2; 0·0309 H_2O,
0·20 g.; 0·0221 N (Kjeldahl),
0·10 g.; 0·0753 AgBr.

	C	H	N	Br
Calculated for $C_6H_7O_4N_2Br$	28·7	2·78	11·1	31·8
Found	29·0	2·74	11·0	32·0

Hydantoin-β-bromopropionic acid is readily soluble in hot water, sparingly in cold water, but dissolves more easily in acetic acid. It is sparingly soluble in ether or chloroform. On ignition, the vapours do not give the pyrrole reaction with a pine splinter, but if the substance is mixed either with sodium carbonate or zinc dust before ignition, an intense reaction is obtained. The whole of the bromine is readily removed on warming for a few minutes with strong sodium hydroxide solution, but it is decidedly more stable towards ammonia. On heating with excess of pyridine at 100°, carbon dioxide is split off together with the bromine, and a neutral product, melting at over 270°, results. Its reaction towards boiling water is described in a subsequent section and the action of barium hydroxide has already been referred to. The neutral salts are not particularly unstable, for a small amount of the acid, digested on the water-bath for half an hour with a suspension of chalk in water, lost only about a third of its bromine.

Hydantoin-β-bromopropionic acid (1 part) was reconverted into hydantoin-propionic acid by warming it on the water-bath with granulated zinc (3 parts) and 50 % acetic acid (10 parts). The solution was evaporated to dryness, dissolved in water and the zinc removed with hydrogen sulphide. On concentrating the filtrate, the hydantoin-propionic acid readily crystallised.

Hydantoin-$\beta.\gamma$-propenylic acid. This acid was separated from the mixture of crystalline substances resulting from the bromination in acetic acid solution of hydantoin-propionic acid, as described in the preceding section. The mixture (5 g.) was boiled for one hour with zinc dust (20 g.) and water (400 cc.). The bulk of the hydantoin-β-bromopropionic acid is completely decomposed by this treatment and the remainder reduced to the very soluble hydantoin-propionic acid, while the unsaturated acid is substantially unaffected. On filtering and concentrating the filtrate to small bulk, a deep yellow zinc salt, moderately soluble in water but insoluble in alcohol, crystallises out. This was filtered off, dissolved in hot water, the zinc removed with hydrogen sulphide and the filtrate again concentrated. About 0·7 g. of a sulphur yellow compound, crystallising in fine hexagonal prisms, was obtained. It is readily soluble in hot water and fairly soluble even in cold water. It melts at 222–223° without darkening and gives a solid sublimate. It gives a strong cherry red colour with diazobenzenesulphonic acid in the presence of sodium hydroxide, thus resembling glutaconic acid, of which it may be regarded as a derivative. On warming with silver nitrate and ammonia, very little or no reduction takes place. On treatment with β-naphthol and excess of concentrated sulphuric acid it gives a clear yellowish green solution in the cold, turning suddenly violet red on gentle warming. The pyrrole reaction is positive. The acid re-

duces permanganate freely in sodium carbonate solution, and absorbs bromine or hydrobromic acid in acetic acid slowly in the cold but readily at about 80°.

Analysis. 0·1598 g.; 0·2468 CO_2; 0·0512 H_2O.

	C	H
Calculated for $C_6H_6O_4N_2$ ...	42·3	3·53
Found	42·1	3·55

Hydantoin-β-chloropropionic acid. This compound was obtained by warming in a sealed tube at 95° hydantoin-propionic acid (8·6 g.) dissolved in fuming hydrochloric acid (40 cc.) and bromine (8·0 g.). The whole of the bromine disappeared in less than an hour. When the tube was kept in a cold place for a couple of days about 3 g. of crystals separated, and further small amounts of less pure material were obtained on concentrating the mother liquor under reduced pressure. The substance was purified by recrystallisation from 20 % hydrochloric acid, in which it is readily soluble when hot but sparingly soluble in the cold. It crystallises in rectangular plates which are without colour when pure. On recrystallising from water they become yellowish. The reactions of the substance are identical with those of the corresponding bromine compound already described. Using litmus paper as indicator, the substance reacts as a monobasic acid—0·2 g. required 9·77 cc. of $N/10$ sodium hydroxide equivalent to a molecular weight of 206—the theoretical value being 206·5.

Analysis. 0·1299 g.; 0·1660 CO_2; 0·0381 H_2O,
0·20 g.; 0·0266 N (Kjeldahl),
0·10 g.; 0·0682 AgCl.

	C	H	N	Cl
Calculated for $C_6H_7O_4N_2Cl$	34·9	3·39	13·5	17·2
Found	34·9	3·26	13·3	16·9

Hydantoinacrylic acid. Hydantoin-β-bromopropionic acid (10 g.) was boiled with water (200 cc.) under a reflux condenser for three or four hours. The solution was then concentrated to about 50 cc. and allowed to crystallise. The yellow crystals thus separated were again boiled for a short time with sufficient fresh distilled water to dissolve them and allowed to crystallise again. On diluting the first mother liquor, again boiling and concentrating to small bulk, a little more of the crystalline product may be obtained. The total yield amounts to 15–20 % of the theoretical. It should be tested for the absence of bromine and if any is found it should be again boiled with water, or alternatively it may be dissolved in excess of warm sodium hydroxide and then precipitated with hydrochloric acid. The substance when pure is sparingly soluble in cold water and only moderately so in hot water. It crystallises in rosettes of sulphur yellow needles melting at 256–258° with effervescence. It is very sparingly soluble in acetic acid, alcohol or ethyl acetate. It does not react with phenylhydrazine acetate but reduces ammoniacal silver solutions on boiling. It does not reduce mercuric chloride. Bromine and hydrobromic acid in acetic acid solution are absorbed slowly when cold, but readily on warming. Diazobenzenesulphonic acid gives a strong cherry red reaction with

a trace of the acid dissolved in sodium hydroxide. The substance reacts as a monobasic acid to litmus—0·1427 g. required 8·8 cc. $N/10$ sodium hydroxide, though the end point was not perfectly sharp. This titration is equivalent to a molecular weight of 162 compared with a calculated value of 170.

Analysis. 0·1405 g.; 0·2179 CO_2; 0·0480 H_2O,
0·1425 g.; 0·0248 N (Kjeldahl).

	C	H	N
Calculated for $C_6H_6O_4N_2$ ...	42·3	3·53	16·5
Found	42·1	3·81	16·7

β-Hydroxyglutamic acid. Hydantoinacrylic acid (2 g.) was boiled under a reflux condenser with recrystallised barium hydroxide (20 g.) and water (60 cc.). At the top of the condenser a small absorption bulb was placed containing standard sulphuric acid and a little alizarin as indicator. Whenever the contents of the bulb became alkaline owing to absorption of ammonia, 1 cc. more normal acid was added. In this way it is possible to follow the course of the hydrolysis and to stop the heating after ammonia has been liberated equivalent to half the nitrogen of the hydantoinacrylic acid. From 5–6 hours were usually sufficient to complete the reaction and more prolonged boiling is disadvantageous. The solution in the flask, which was turbid from separation of barium carbonate but was no longer yellow, was made acid to Congo-red with sulphuric acid and filtered from barium sulphate. The solution was then concentrated to about 20 cc. on the water-bath and while still acid to Congo-red silver nitrate (5 g.) added. The small precipitate containing traces of silver chloride was filtered off and the silver salt of the amino-acid was precipitated from the filtrate as previously described for the natural acid [Dakin, 1918]. It is important to use pure alkali especially free from silica. The silver salt mixed with excess of silver oxide was filtered off and thoroughly well washed with cold water. It was then suspended in water (20 cc.) and completely decomposed with hydrogen sulphide. The filtrate from the silver sulphide was then concentrated to about 1 cc. in volume at a temperature of not over 50°. Absolute alcohol in excess was then added to the syrup with stirring and the sticky mass of amino-acid thoroughly well washed with alcohol. It was then dissolved in a few drops of cold water, in which it dissolves with the greatest ease, and reprecipitated with alcohol. The acid separates out as a white granular powder which is apt to be sticky if much water is present. It is dried *in vacuo* over phosphorus pentoxide at room temperature since it very readily passes over to hydroxypyrrolidonecarboxylic acid on heating. It was not found possible to crystallise the acid satisfactorily and its solutions when allowed to evaporate *in vacuo* gave a brittle glassy mass. The yield of amino-acid from 2 g. of hydantoinacrylic acid varied from 0·3–0·4 g.

Like the natural acid, the synthetic product gives the pyrrole reaction on heating, which is intensified on igniting with zinc dust. On coupling with diazobenzenesulphonic acid in sodium carbonate solution an orange red colour is obtained, while with sodium hydroxide as alkali, a deep cherry red is

obtained which is intensified on heating. The reaction is sensitive to about 1 : 1000. On warming with β-naphthol and excess of concentrated sulphuric acid a greenish-yellow fluorescent solution is obtained similar to that given by malic acid (Pinerua). Its conversion through loss of water on heating to 105° into a hydroxypyrrolidonecarboxylic acid is also characteristic.

As stated earlier, a small amount of the amino-acid may be obtained by the direct decomposition of hydantoin-β-bromopropionic acid with barium hydroxide, but by far the larger part of the acid undergoes profound decomposition with rupture of the carbon chain and the yield of amino-acid is extremely small. Better results may be obtained by hydrolysing the hydantoin-β-bromopropionic acid (5 g.) by heating in a sealed tube for four to five hours with five parts of fuming hydrochloric or hydrobromic acid. The contents of the tube are evaporated under reduced pressure, milk of lime is then added until the acid reaction to Congo-red is almost but not quite abolished and the diluted mixture (200 cc.) is then boiled for a couple of hours. Excess of lime (5 g.) is then added and boiling resumed until ammonia is no longer evolved. Excess of lime is removed by a current of carbon dioxide and the filtrate containing the calcium salt of the amino-acid is concentrated at low temperature to a volume of about 10 cc. The calcium salt is then precipitated by alcohol, leaving the bulk of the calcium chloride or bromide in solution. The white, very soluble calcium salt is dissolved in water, and the calcium removed exactly with sodium oxalate. Residual traces of chlorides and bromides are removed with silver nitrate after acidifying with a little sulphuric acid and then the amino-acid is precipitated as silver salt as previously described and the latter decomposed with hydrogen sulphide. The concentrated aqueous solution of the amino-acid is twice precipitated with alcohol as described in the preceding paragraph. The acid thus obtained appears to be identical with that from hydantoinacrylic acid.

In some cases, an additional precipitation of the acid was effected by precipitation of its aqueous solution with mercuric acetate in feebly acid or neutral solution. The white mercury precipitate was washed with water, then with alcohol and finally decomposed with hydrogen sulphide in the usual way. Occasionally this procedure is useful for removing traces of inorganic impurities.

For analysis, the acid was dried at room temperature over phosphorus pentoxide at 2 mm. pressure.

Analysis. 0·1979 g.; 0·2670 CO_2; 0·1008 H_2O,
0·1022 g.; 0·1399 CO_2; 0·0483 H_2O,
0·0100 g.; 1·46 cc. N at 17° and 752 mm. (van Slyke).

	C	H	N
Calculated for $C_5H_9O_5N$ ...	36·8	5·52	8·59
Found	36·8	5·66	8·34
	37·3	5·57	

On heating the acid either under normal or reduced pressure, water is evolved

freely in the neighbourhood of 100° and the substance is converted mainly into hydroxypyrrolidonecarboxylic acid. Thus on heating 46·2 mg. of the acid for one hour at 105–107°, the product on treatment with nitrous acid gave only 0·48 cc. nitrogen, equivalent to 0·58 % of nitrogen in the NH_2 form, indicating that about 93 % of the acid had been decomposed. An analysis of such a dried product is appended:

Analysis. 0·1168 g.; 0·1783 CO_2; 0·0542 H_2O.

	C	H
Calculated for $C_5H_7O_4N$ ...	41·3	4·9
Found	41·7	5·02

The copper salt of i-*β-hydroxyglutamic acid* is a clear blue-green salt which is very soluble in water but insoluble in alcohol. It resembles the copper salt of the natural active acid except that it appears a shade less blue and more green. It was prepared in the usual way by digesting an aqueous solution of the acid with excess of well-washed copper hydroxide. After filtering off the excess of copper oxide, the solution should be evaporated at a low temperature, as prolonged heating is apt to cause the separation of a little copper oxide. The concentrated aqueous solution of the copper salt was precipitated with alcohol and dried first over sulphuric acid and then in the oven at 100°.

Analysis. 0·1681 g.; 0·1591 CO_2; 0·0497 H_2O; 0·0566 CuO, 0·0200 g.; 2·1 cc. N at 13° and 752 mm. (van Slyke).

	C	H	N	Cu
Calculated for $C_5H_7O_5NCu$	26·7	3·12	6·23	28·3
Found	26·0	3·28	6·12	27·7

The calcium salts of i-*β-hydroxyglutamic acid* are very soluble in water but insoluble in alcohol. They are precipitated as white powders on adding alcohol to their aqueous solutions. The acid calcium salt was obtained by warming the acid with calcium carbonate in water and was found to contain 11·3 % calcium (calculated 11·0). The "neutral" salt which is strongly alkaline in reaction towards litmus, was obtained by using excess of milk of lime and precipitating with alcohol. It contained 20·0 % calcium (calculated 19·9). It is decomposed, but incompletely, by passing carbon dioxide into its aqueous solution.

The strychnine salt of i-*β-hydroxyglutamic acid* was prepared by warming a concentrated solution of the acid with twice its weight of strychnine and adding methyl alcohol (10 parts). When all the base had dissolved, the water and alcohol were removed under diminished pressure and the sticky residue was crystallised from ten parts of boiling butyl alcohol. On standing, rosettes of needles separated out, which were readily soluble in water or methyl alcohol, but less so in ethyl or butyl alcohol.

Analysis. 0·1192 g.; 0·2750 CO_2; 0·0666 H_2O.

	C	H
Calculated for $C_{26}H_{31}O_7N_3$...	62·8	6·24
Found	62·9	6·21

Malic semi-aldehyde.

Malic semi-aldehyde or β-hydroxy-γ-aldehydobutyric acid was obtained by the hydrolysis of its acetal, γ-diethoxy-β-hydroxybutyric acid, which was in turn obtained by the reduction of γ-diethoxyacetoacetic ester already prepared by Dudley and the writer [1914].

γ-Diethoxy-β-hydroxybutyric acid. Ethyl γ-diethoxyacetoacetate (21·8 g.) together with alcohol (20 cc.) were mixed with 180 cc. of water. The mixture was surrounded by an ice bath and thoroughly agitated with a mechanical stirrer. 4 % sodium amalgam (200 g.) was added fairly rapidly and but little gas was evolved until the reduction was complete at the end of about seven hours. The mixture was allowed to stand overnight and then saturated with carbon dioxide and evaporated almost to dryness under reduced pressure. In the earlier experiments the sodium salt of the hydroxy-acid was extracted by hot methyl alcohol in which it is freely soluble, but later this step was avoided as follows: 25 % sulphuric acid was added to the thick residue until the mixture was acid to Congo-red and an oily layer separated out. The oil was at once extracted with ether and the aqueous layer again shaken with ether. The ether extract was washed with a few drops of water, dried over sodium sulphate and evaporated. The oil which weighed about 14 g. after drying over sulphuric acid was practically pure as judged by analysis. On distilling the acid under reduced pressure, there is a good deal of decomposition; about half of it is obtained as a clear oil boiling at 120° under 5 mm. pressure.

Analysis. 0·1558 g.; 0·2860 CO_2; 0·1186 H_2O,
0·1285 g. (distilled); 0·2341 CO_2; 0·0935 H_2O.

	C	H
Calculated for $C_8H_{16}O_5$ ...	50·0	8·33
Found	50·1	8·45
„	49·7	8·08

The acid is a sour smelling oil suggestive of butyric and hydroxybutyric acids. It mixes readily with a little water but is precipitated on diluting, finally again dissolving in about six parts of cold water. It does not decolorise bromine even after distillation and is readily soluble in ether and chloroform, and to a noticeable extent in petroleum ether. The salts are almost all very soluble and do not crystallise easily. The *sodium salt* soluble in methyl alcohol has already been referred to. The *zinc salt* is very soluble and is not precipitated by alcohol. When its solutions are evaporated in a desiccator, it is obtained as clear brittle semi-crystalline flakes which melt at a low temperature. On adding silver nitrate to a 3 % solution of the sodium salt, no precipitation occurred in the cold but, on warming, prompt reduction took place. The acid when titrated with alkali, using phenolphthalein as indicator, gave the following result: 0·2850 g. neutralised 15·0 cc. $N/10$ sodium hydroxide, giving a molecular weight of 190 compared with a calculated value of 192.

Malic semi-aldehyde. γ-Diethoxy-β-hydroxybutyric acid (15 g.) prepared as just described was gently boiled with 50 cc. of 0·2 N sulphuric acid for

about an hour. After a few minutes, the whole of the oil dissolved. An amount of barium hydroxide exactly equivalent to the sulphuric acid was then added and the barium sulphate removed by filtration. The filtrate was evaporated under reduced pressure at not over 45°, when a clear syrup, readily soluble in water and alcohol but sparingly soluble in ether or chloroform, was left behind. It was impossible to distil the substance without decomposition and it did not crystallise. The yield of dry syrup was 8 g. It is inadvisable to treat the substance with strong alcohol for any length of time since either acetal or ester formation takes place easily. A portion was however dissolved in alcohol, filtered from a trace of inorganic impurities, at once diluted with water and then concentrated. It was finally dried over phosphorus pentoxide *in vacuo* and analysed with the following result:

Analysis. 0·1454 g.; 0·2185 CO_2; 0·0721 H_2O.

	C	H
Calculated for $C_4H_6O_4$ ...	40·7	5·09
Found	41·0	5·51

The complete purity of the product can hardly be anticipated since it could be neither distilled nor crystallised, but the results indicate no great amount of impurity. On titrating with alkali, using phenolphthalein as indicator, 0·1560 g. required 12·8 cc. *N*/10 sodium hydroxide, equivalent to a molecular weight of 122 compared with a calculated value of 118.

Malic semi-aldehyde reduces ammoniacal silver solutions on warming and instantly in the cold in the presence of sodium hydroxide. It gives a strong reaction with Schiff's reagent, especially if the reagent is dilute and not excessively acid. Bromine water oxidises the aldehyde freely, and on warming with excess of sodium hydroxide it gives inactive malic acid which was identified through its lead salt. It reacts with phenylhydrazines, but with the exception of *p*-nitrophenylhydrazine the osazones formed are not very attractive. On mixing the aldehyde in aqueous solution with semicarbazide hydrochloride, preferably without addition of alkali, a good yield of *semicarbazone* is obtained crystallising in prisms melting at 211° with effervescence.

Analysis. 0·1287 g.; 0·1630 CO_2; 0·0580 H_2O.

	C	H
Calculated for $C_5H_9O_4N_3$ ...	34·3	5·14
Found	34·5	5·00

Action of ammonia and hydrocyanic acid on malic semi-aldehyde. The attempts to utilise Strecker's method for the synthesis of β-hydroxyglutamic acid were made with 10 % alcoholic solutions of the aldehyde, adding anhydrous hydrocyanic acid (1 mol.) together with a few drops of alcoholic ammonia and then adding alcoholic ammonia (2 mols.) on the second day. The conditions were substantially those used by Fischer in his synthesis of serine. On the third day, fuming hydrochloric acid was added for the purpose of hydrolysing any amino-cyanohydrin that might be formed. In other experiments, the ammonia was added first and the hydrocyanic acid later, but the

results were no better. After saturating with hydrochloric acid and boiling for an hour, the mixture was evaporated to dryness and the residue extracted with alcohol, leaving most of the ammonium chloride behind. The alcohol was then removed by evaporation and the solution made alkaline with sodium carbonate and boiled to remove ammonia. The solution was then just acidified with acetic acid and precipitated with mercuric acetate. The small precipitate was decomposed with hydrogen sulphide and any β-hydroxyglutamic acid was isolated as silver salt after removing traces of chlorides. 10 g. of the aldehyde yielded about 0·25 g. of silver salt equivalent to 0·11 g. of acid and on analysis it was found to be decidedly impure. It gave, however, the qualitative reactions for β-hydroxyglutamic acid previously described, so that it is not improbable that a minute amount of the amino-acid had been formed. But it was clear that at any rate in its present form the synthesis was not a practicable one.

Experiments with derivatives of acetonedicarboxylic ester.

The direct reduction of α-isonitroso-acetonedicarboxylic ester gave unsatisfactory results, as already stated. The nitroso-compound was prepared in large quantities according to Pechmann's [1891] directions, and practically every reducing agent that had any prospect of success was tried. The reduction in either acid or neutral media gave products that on analysis furnished results similar to those required for β-hydroxyglutamic acid or its anhydride, but the product was mostly not an amino-acid as judged by the nitrous acid reaction, nor did it give an amino-acid as the main product on warming with mineral acids. For reasons already given, it was decided to attempt to lessen the reactivity of the carbonyl group of the acetonedicarboxylic ester and its nitroso derivative by condensation with orthoformic ester, since as shown in the following section, the ethoxyglutaconates thus formed may be reduced by sodium amalgam to β-hydroxyglutaric acids.

Reduction of β-ethoxyglutaconic ester. Diethyl β-ethoxyglutaconate was prepared by the condensation of acetonedicarboxylic ester and orthoformic ester with acetyl chloride as described by Claisen (cp. D.R.P. 80739) and the product was fractionated under reduced pressure. The ester (10 g.) was mixed with alcohol (75 cc.) and water (75 cc.) and then reduced with 4 % sodium amalgam (100 g.) which was added rapidly. The reaction, which was slow, was carried on at first in an ice bath with constant stirring, but later at room temperature. After standing overnight, phenolphthalein was added and the solution exactly neutralised with nitric acid. The filtered solution was then precipitated with excess of silver nitrate solution and the silver salt washed with water. A portion of the silver salt on ignition was found to contain 59·0 % of silver compared with 59·6 for silver β-hydroxyglutarate and 55·4 % for silver β-ethoxyglutarate. That β-hydroxyglutaric acid was the main or sole product of the reaction was proved by decomposing the remaining silver salt suspended in water with hydrogen sulphide, and evaporating the filtrate. The residue

crystallised completely and, after spreading on a porous plate and washing with a little ether, melted at 93–95°. The yield was 55 % of the calculated. The β-hydroxyglutaric acid was further identified by its *bis*-phenylhydrazide prepared by warming the acid with three parts of phenylhydrazine for 15 minutes at 120°. The substance crystallised from glacial acetic acid in fine white needles melting at 234–235° as described by Pechmann. The free acid was also analysed:

Analysis. 0·1265 g.; 0·1878 CO_2; 0·0623 H_2O.

	C	H
Calculated for $C_5H_8O_5$ ...	40·5	5·41
Found	40·5	5·47

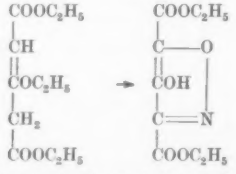
α-Nitroso-β-ethoxyglutaconic ester (?). On adding ethyl orthoformate (1 mol.) to α-nitroso-acetonedicarboxylic ester (1 mol.) no obvious reaction takes place, but on adding an acid catalyst, such as acetyl chloride or a little alcoholic hydrochloric acid, a vigorous reaction occurs with liberation of heat. On warming to 60° under diminished pressure, ethyl formate and, when acetyl chloride has been used, ethyl acetate are found in the distillate. The residual product is apparently a mixture of the α-nitroso-β-ethoxyglutaconic ester with unchanged α-nitroso-acetonedicarboxylic ester, as judged by the results of elementary analysis. The former compound requires 50·9 % carbon, 6·57 % hydrogen, the latter requires 46·7 % carbon and 5·63 % hydrogen. The observed results varied from 46·0–50·1 % carbon and 5·65–6·61 % hydrogen. It would therefore appear as if the ethoxy-compound were readily reconvertible into the ketonic ester.

The ease with which the ethyl group of ethoxyglutaconic ester could be eliminated on reduction has been shown in the foregoing. A further example is furnished by the action of excess of amyl nitrite (2 mols.) on β-ethoxyglutaconic ester (1 mol.) in the presence of 2 % of alcoholic hydrochloric acid. A crystalline product was readily separated which proved to be hydroxyisoxazoledicarboxylic ethyl ester. It crystallised from alcohol in needles melting at 104° as described by Pechmann.

Analysis. 0·1835 g.; 0·3162 CO_2: 0·0820 H_2O.

	C	H
Calculated for $C_9H_{11}O_6N$ ...	47·0	4·80
Found	47·2	4·96

The reaction may be represented as follows:

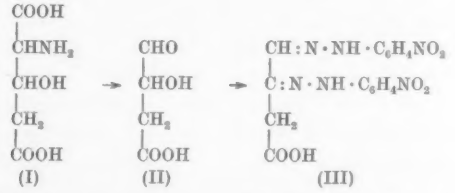


The preceding reactions clearly indicated the difficulty that was likely to be encountered in obtaining pure α-nitroso-β-ethoxyglutaconic ester starting

either from ethoxyglutaconic ester or α-nitroso-acetonedicarboxylic ester. Accordingly the products of the interaction of α-nitroso-acetonedicarboxylic ester (1 mol.) and orthoformic ester (1 mol.) after standing with a trace of alcoholic hydrochloric acid for 24 hours at room temperature were reduced in 50 % alcoholic solution without further purification, using a large excess of sodium amalgam at 0°. The reaction was kept feebly acid with sulphuric acid at first and later was allowed to become alkaline. A current of carbon dioxide was passed during the reaction which lasted 12–18 hours. As stated in the introduction, the isolation of the products of the reaction is difficult and has not been satisfactorily solved. The separation of the products by precipitating with mercuric acetate and then isolating the silver salt of the acid contained in the mercury precipitate gave a product which resembled hydroxyglutamic acid in its properties but was accompanied by other substances closely similar in elementary composition. It may be mentioned that a small amount of apparently pure β-hydroxyglutamic acid and its copper salt have been isolated with great difficulty, but much further work is needed to clear up the course of the reaction and the sharp separation of the products. The study of the reaction will be continued. The purest specimen of acid hitherto obtained by the foregoing method contained 36·4 % carbon, 5·63 % hydrogen and 8·4 % amino-nitrogen. The copper salt, which was greenish-blue resembling that obtained by alternative methods, contained 29·4 % of copper and 6·15 % nitrogen. These results are in fair agreement with the calculated figures. But this method of synthesis in its present form is much less satisfactory than that from hydantoin-propionic acid previously described.

II. The *p*-nitrophenylosazones of malic semi-aldehyde and tartronic semi-aldehyde.

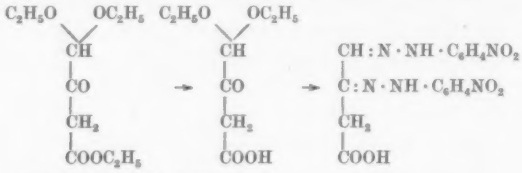
One of the main pieces of evidence which led the writer to assign to the amino-acid separated from caseinogen [1918] the structure of β-hydroxyglutamic acid was the fact that on oxidation it gave an aldehyde (II) which reacted with *p*-nitrophenylhydrazine to give a well-defined osazone (III), thus indicating that the amino and hydroxyl groups were attached to contiguous carbon atoms.



It appeared desirable, in order to confirm this deduction, to prepare the osazone in some entirely independent fashion, and this has now been done and the apparent identity of the products established. The preparation of its

lower homologue is also described, a substance which has since been obtained by the oxidation of hydroxyaspartic acid. The synthesis of the latter amino-acid from chloromalic acid will be described in a separate communication.

Malic semi-aldehyde p-*nitrophenylosazone.* γ-Diethoxyacetoacetic ester [Dakin and Dudley, 1914] (3 g.) was shaken with 15 cc. of normal sodium hydroxide at room temperature and the mixture allowed to stand overnight. The solution was then neutralised with dilute sulphuric acid and unchanged ester removed by extracting twice with ether. The aqueous solution was then made decidedly acid to Congo-red with more sulphuric acid and nitrophenylhydrazine (3 g.) dissolved in 15 cc. of glacial acetic acid was added. An immediate precipitate was formed and the reaction was completed by heating for 20 minutes on the water-bath. The precipitate was filtered off, washed well with warm methyl alcohol and recrystallised from boiling nitrobenzene. The yield was 1·0 g. The reactions may be represented as follows:



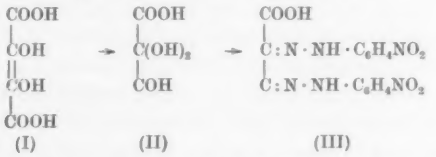
Analysis. 0·1856 g.; 0·3470 CO_2; 0·0650 H_2O.

	C	H
Calculated for $C_{16}H_{14}O_6N_6$...	51·1	3·72
Found	51·0	3·89

The *osazone* is sparingly soluble in most organic solvents but it dissolves very readily in boiling nitrobenzene, crystallising out on cooling in the form of deep brown-red thick prismatic needles. It dissolves readily in pyridine even in the cold forming a salt, but is practically insoluble in water, alcohol, ether or chloroform. Its melting point varies considerably with the rate of heating and, if this is slow, may be as low as 275°, while with rapid heating it is 289–291° (uncorr.) or 297–299° (corr.). Its melting point was unchanged on mixing it with the osazone prepared from natural β-hydroxyglutamic acid as described previously [Dakin, 1918]. On adding alcoholic sodium hydroxide to a trace of the substance, a clear blue coloration is obtained, characteristic of two adjacent nitrophenylhydrazine groups.

Tartronic semi-aldehyde p-*nitrophenylosazone.* A solution of tartronic semi-aldehyde (II) was obtained according to Fenton's [1905] directions. Dihydroxymaleic acid (I) (1·48 g.) was added to mercuric chloride (5·5 g.) in water (15 cc.) and the mixture kept at 65–70° for an hour. The solution was then filtered from calomel and the residual mercury removed with hydrogen sulphide. Excess of hydrogen sulphide was removed by exposure to reduced pressure and then nitrophenylhydrazine (3·5 g.) dissolved in hot glacial acetic acid (15 cc.) was added. An immediate red precipitate was formed and the mixture was warmed for 20 minutes on a steam bath. The osazone (III) was

then filtered off and well washed with warm methyl alcohol. The yield was 2·2 g. The reactions may be represented as follows:



(I) (II) (III)

The *osazone* is sparingly soluble in alcohol, ether or chloroform, but readily dissolves in pyridine with formation of a salt which does not readily separate out. The osazone can be recrystallised from boiling nitrobenzene but it is much less soluble than the preceding osazone and the crystalline form is quite different. It separates from nitrobenzene solution in nodular rosettes of fine needles with a few separate long fine needles. The melting point of the recrystallised substance is about 302° uncorr. (310° corr.) with effervescence. Slight darkening occurs above 270°. A trace of the substance dissolved in alcoholic sodium hydroxide with an intense clear blue colour.

Analysis. 0·1780 g.; 0·3192 CO_2; 0·0570 H_2O.

	C	H
Calculated for $C_{15}H_{12}O_6N_6$ ...	48·9	3·23
Found	48·9	3·56

An identical osazone has been obtained from the oxidation products of synthetic hydroxyaspartic acid, under the same conditions as those used for the oxidation of β-hydroxyglutamic acid.

III. Optical activity of natural β-hydroxyglutamic acid.

In the first communication on β-hydroxyglutamic acid [Dakin, 1918] from caseinogen it was stated that "in aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid." The difficulties in obtaining trustworthy observations on the optical activity of the acid are mainly two-fold. In the first place, the difficulty of satisfactorily recrystallising the acid and the fact that its purification as then described rested almost as much on methods for the removal of other substances as for the direct separation of the acid in some characteristic form. The second difficulty was not appreciated at the beginning of the work and was the cause of considerable confusion until it was established that *d*-β-hydroxyglutamic acid passed over into a laevorotatory hydroxypyrrolidonecarboxylic acid on evaporating its aqueous solution or drying at 100°.

Accordingly, experiments have been made on the rotation of specimens of β-hydroxyglutamic acid, two of which were prepared from the repeatedly crystallised strychnine salt to be described in the next section, while one was obtained from the products of tryptic digestion, and all of them were carefully handled at low temperature to avoid formation of the laevorotatory anhydride. The observations are probably fairly accurate, though the possibility of the need of revision is by no means excluded.

It was found with all specimens that the free acid had a very slight dextro-rotation which was much increased in the presence of hydrochloric acid. Further, a dextro solution of the free acid on evaporation and drying at 105–110° gave a strongly laevorotatory solution which could be changed back to dextro on treating with hydrochloric acid—the first change in rotation on drying being due to the formation of hydroxypyrrolidonecarboxylic acid and the second to its reconversion into hydroxyglutamic acid. No apparent racemisation accompanied these changes. Alkaline solutions of the potassium or sodium salts of β-hydroxyglutamic acid were found to be practically inactive, but on adding Walden's uranium solution, a fairly strong laevo-rotation was observed. This behaviour shows a close analogy to malic acid and other hydroxy-acids.

Specimen A was obtained as described in a previous paper [Dakin, 1918] and was redissolved in 50 % alcohol, precipitated with mercuric acetate in feebly acid solution and the hydroxyglutamic acid obtained by decomposing the washed precipitate with hydrogen sulphide and evaporating at low temperature (25–30°). It crystallised readily and on combustion gave 37·0 % carbon and 5·57 % hydrogen. (Calculated 36·8 % carbon and 5·52 % hydrogen.)

Specimen B was obtained by the same methods as previously employed, but the caseinogen, instead of being hydrolysed with sulphuric acid, was thoroughly digested with trypsin. The yield was disappointing, only 4·5 g. being obtained from 350 g. caseinogen.

Specimens C and D were prepared similarly to A, and then converted into the strychnine salt (see next section) which was repeatedly crystallised and then decomposed with sodium carbonate. The strychnine was filtered off, and traces remaining in solution removed by shaking with amyl alcohol. The acid was then removed by precipitation with mercuric acetate in feebly acid solution, followed by decomposition with hydrogen sulphide. The polarimetric observations are recorded in the following table:

Conditions	Specimen	Concentration	Length of tube, dm.	Observed rotation	$[\alpha]_D^{20°}$
Free acid in aqueous solution	A	5·43	2·2	+0·05°	+ 0·4°
" " "	B	3·0	1·0	+0·02	+ 0·7
" " "	C	4·0	2·0	+0·08	+ 1·0
" " "	D	4·0	2·0	+0·10	+ 1·2
Acid in 20 % hydrochloric acid	A	2·0	2·0	+0·62	+15·5
" " "	B	3·0	1·0	+0·48	+16·0
" " "	C	1·33	2·0	+0·43	+16·2
" " "	D	1·33	2·0	+0·47	+17·6
Acid dissolved in *N* potassium hydroxide	B	3·0	1·0	0	—
" " " "	C	2·0	2·0	0	—
Filtered solution from mixture containing 0·4 *N* potassium hydroxide and 12 % uranium nitrate	A	1·08	1·0	−0·13	−12·0
Ditto, 0·2 *N* potassium hydroxide, and 20 % uranium nitrate	C	0·60	2·2	−0·32	−24·3
Acid heated 2 hours 105°	A	1·73	2·0	−0·37	−10·9
" 2 "	B	1·0	1·0	−0·15	−15·0
" 4 "	C	1·20	2·0	−0·27	−11·3
* Solution C after heating with 20 % hydrochloric acid	C*	1·20	1·0	+0·20	+16·6

Taking a mean of the various observations, it may be stated that the apparent specific rotation of the free acid in 4 % solution is about + 0·8°, while a 2 % solution in 20 % hydrochloric acid has a rotation of + 16·3°. The potassium salt apparently has a negligible rotation but is strongly laevorotatory on addition of uranium nitrate. The acid becomes strongly laevorotatory on drying at 105°, but the numbers have no great quantitative value since the conversion of the hydroxyglutamic acid into its anhydride is not known to be perfectly complete.

IV. Alkaloid salts of β-hydroxyglutamic acid and related acids.

One of the main difficulties in working with hydroxyglutamic acid lay in the paucity of simple derivatives, capable of purification by repeated crystallisation. The metallic salts are either very soluble substances that require precipitation by alcohol or insoluble precipitates that are incapable of recrystallisation. It therefore became necessary to search for other salts with more acceptable properties. Of the common alkaloids only the salts of strychnine and brucine proved to be of value since the others mostly underwent hydrolytic dissociation too easily. For purposes of comparison, the corresponding salts of aspartic, glutamic and pyrrolidonecarboxylic acid have been prepared. Many of these salts are finely crystalline products and can be readily recrystallised from appropriate solvents but it is to be regretted that their melting points are not sharp in most cases, though their behaviour on heating is often characteristic.

All the salts contained equimolecular proportions of base and acid and were prepared by dissolving the acid in warm water, then adding the alkaloid till the solution became neutral to litmus, and adding a little methyl alcohol from time to time to aid in the solution of the base. The neutral solution was then evaporated at low temperature to avoid hydrolytic decomposition and recrystallised from an appropriate solvent. The specific rotation of the salts was determined in aqueous solution.

Strychnine d-*β-hydroxyglutamate.* The salt is very soluble in water and on slow evaporation of its solution separates out in the form of nodular masses which are spread on porous plates and recrystallised. Butyl alcohol containing a little water has been found to be decidedly the best solvent for this purpose. Methyl alcohol dissolves the salt too readily, while the solubility in ethyl alcohol is not much greater at the boiling temperature than at room temperature. 5 g. of the strychnine salt require about 60 cc. of butyl alcohol and will give about 3·5 g. of fine prismatic needles. The composition of the salt does not change on repeated crystallisation nor does its behaviour on heating. As observed in a melting point tube, a change takes place fairly sharply at a temperature varying from 165 to 175°, giving a white opaque waxy mass which does not melt till about 245°, when it gives a clear brown oil without effervescence. On dissolving the salt crystallised from butyl alcohol in water, no strychnine separates, and on evaporation long striated needles of the salt

crystallise out. These contain practically no water of crystallisation (0·3 %) and show the same behaviour on heating as that just described. The strychnine salt of β-hydroxyglutamic acid is preferable to any of the others for purposes of purification and characterisation of the acid.

Specific rotation. C = 1·67; l = 2 dm.; $\alpha = -0{\cdot}88°$; $[\alpha]_D^{20°} = -26{\cdot}3°$.

Analysis. 0·1629 g.; 0·3760 CO_2; 0·0963 H_2O,
0·1145 g.*; 0·2640 CO_2; 0·0630 H_2O.
* Recrystallised five times.

	C	H
Calculated for $C_{26}H_{31}O_7N_3$	62·8	6·24
Found	62·9	6·57
„	62·9	6·11

Brucine d-*β-hydroxyglutamate.* This salt separates out in the form of needles containing water of crystallisation on evaporation of its aqueous solution at low temperatures. It is best purified by spreading on a porous tile, then warming with acetone in which it is insoluble and recrystallising the residue either from a little water or from methyl alcohol. The salt crystallised from water begins to soften at about 90° and melts to a turbid fluid around 110°. The anhydrous salt obtained as prismatic needles by crystallisation from dry methyl alcohol decomposes indefinitely above 200°. Both salts are extremely soluble in water and alcohol but almost insoluble in acetone.

Specific rotation. C = 1·0; l = 2·0; $\alpha = -0{\cdot}50°$; $[\alpha]_D^{20°} = -25{\cdot}0°$.

Analysis. 0·1540 g.; 0·3368 CO_2; 0·0905 H_2O.

	C	H
Calculated for $C_{28}H_{35}O_9N_3$	60·3	6·26
Found	59·6	6·53

Strychnine d-*glutamate.* The salt is prepared as usual but the neutral solution should be concentrated at low temperatures and methyl alcohol added by degrees to replace the water. A syrupy mass results which, on mixing with excess of absolute alcohol, gives stout isolated glistening prisms which dissolve in water without separation of strychnine. The salt is practically insoluble in dry methyl, ethyl or butyl alcohols, but extremely soluble in water. It begins to show signs of decomposition on heating above 200° and melts about 225–230°.

Specific rotation. C = 2·0; l = 2·0; $\alpha = -1{\cdot}02°$; $[\alpha]_D^{20°} = -25{\cdot}5°$.

Analysis. 0·2217 g. dried at 100°; 0·0196 N (Kjeldahl) = 8·84 % N.
Calculated for $C_{26}H_{31}O_6N_3$ 8·69 „

Brucine d-*glutamate.* The salt crystallises from water in the form of long stout prismatic needles with a satin lustre. These crystals contain about 15·5 % of water of crystallisation which is lost at 100°. This corresponds to between five (14·2 %) and six molecules (16·4 %) of water. The salt as crystallised from water dissolves readily in hot methyl alcohol and, on cooling, bright rhombic prisms of the anhydrous salt separate out. The water-containing salt dissolves less readily in ethyl or butyl alcohol. After recrystallisation from

methyl alcohol, the anhydrous salt is soluble only with great difficulty in any of the alcohols. The salt containing 5·5 mols. of water softens at about 96° and melts at about 101°, while the anhydrous salt slowly decomposes above 170° but does not melt until about 240°.

Specific rotation. C=2·0 (anhydrous); l=2·0; α= −0·92°; $[\alpha]_D^{20°}$ = −23·0°.

Analysis. 0·2 g. dried at 100°; 0·0154 N (Kjeldahl) = 7·70 % N.
Calculated for $C_{28}H_{35}O_8N_3$ 7·76 „

Strychnine l-*aspartate.* The salt is very soluble in water and separates in crusts which have the appearance of stout prisms to the naked eye but are made up of masses of fine interlacing needles. Its solubilities closely parallel those of the corresponding glutamate and it melts fairly sharply to a brown oil with effervescence around 252–255°.

Specific rotation. C=1·164; l=2·0; α= −0·66°; $[\alpha]_D^{20°}$ = −28·3°.

Analysis. 0·1500 g.; 0·0134 N (Kjeldahl) = 8·90 % N.
Calculated for $C_{25}H_{29}O_6N_3$ 9·0 „

Brucine l-*aspartate.* As first obtained from aqueous solution, this salt forms rather waxy prisms which become hard and brittle on drying and take on an opaque white appearance. It contains 5 molecules of water of crystallisation which are given off at 90°. (Water: found 14·9 %, calculated 14·6.) The salt is readily soluble in methyl alcohol but does not crystallise out readily on cooling. On adding ethyl acetate, in which it is sparingly soluble, it is deposited as fine needles. The anhydrous salt is moderately soluble in ethyl alcohol and less so in butyl alcohol, but does not crystallise out readily. It is insoluble in acetone. The salt containing water of crystallisation softens at 96° and melts about 100°, while the anhydrous salt decomposes indefinitely above 200°.

Specific rotation. C=1·702 (anhydrous); l=2·0; α= −0·97°; $[\alpha]_D^{20°}$ = −28·4°.

Analysis. 0·1500 g.; 0·0121 N (Kjeldahl) = 8·05 % N.
Calculated for $C_{27}H_{33}O_8N_3$ 7·97 „

Strychnine l-*α-pyrrolidone-carboxylate.* The acid used for preparing this and the succeeding salt was obtained by heating glutamic acid under reduced pressure at 165° and then crystallising the products from alcohol. It had a specific rotation in water of − 5·7°, and hence was partially racemised since the pure laevo-acid is generally regarded as possessing a rotation of about − 7·2°.

The salt crystallises from water in the form of felted fan-shaped masses of fine needles which are readily soluble in ethyl alcohol but sparingly soluble in acetone. It dissolves easily in butyl alcohol and also in chloroform but crystallises better from water than from any of the organic solvents. The salt thus obtained softens on heating at 90° and partially melts. It contains water of crystallisation which is lost at 100°. The anhydrous salt melts indefinitely but incompletely until about 245° is reached.

Specific rotation. C=2·74; l=2·0; α= −1·45°; $[\alpha]_D^{20°}$ = −26·7°.

Analysis. 0·2438 g.; 0·0218 N (Kjeldahl) = 8·96 % N.
Calculated for $C_{26}H_{29}O_5N_3$ 9·07 „

Brucine l-*α-pyrrolidone-carboxylate.* This salt is better suited than the preceding one for purposes of characterisation of the acid. On concentrating the neutral aqueous solution, a syrup was obtained which at once solidified to a mass of stout prisms. The salt is very soluble in methyl alcohol but can be conveniently crystallised from boiling acetone from which it separates in well-formed prisms. On heating the substance, decomposition can be noted as low as 140° and it melts with effervescence to a clear fluid from 180–195°.

Specific rotation. $C=5{\cdot}0$; $l=2{\cdot}0$; $\alpha=-3{\cdot}15°$; $[\alpha]_D^{20°}=-31{\cdot}5°$.

Analysis. 0·4619 g.; 0·0364 N (Kjeldahl) = 7·88 % N.
Calculated for $C_{28}H_{32}O_7N_2$ 8·03 ,,

V. Identification of β-hydroxyglutamic acid among the products of hydrolysis of glutenin and gliadin.

The experiments in the following section were made with the cooperation of Miss Marjorie Lord Strauss, to whom acknowledgment is due for much valuable assistance.

Hydroxyglutamic acid up to the present has only been isolated from caseinogen, a typical animal protein, after hydrolysis with acids, with trypsin, and possibly to a smaller extent with barium hydroxide. There seems every reason to suppose that search for this amino-acid will disclose its presence in other proteins. The present communication records its presence in two typical vegetable proteins, glutenin and gliadin.

The protein preparations were prepared from wheat flour according to the excellent methods described by Osborne. The methods employed were practically identical with those described in the work on caseinogen [Dakin, 1918]. Portions of 100 g. of each protein were hydrolysed by prolonged boiling with dilute sulphuric acid and the latter subsequently removed exactly with barium hydroxide. The concentrated solution of amino-acids was then thoroughly extracted with butyl alcohol and the aqueous residue containing essentially the strong acids and bases was saturated with hydrochloric acid to separate the bulk of the glutamic acid. The calcium salts of the acids were then separated by alcohol according to Foreman's method. They were dissolved in water, the calcium removed exactly with oxalic acid, and the diluted filtrate boiled with lead hydroxide to remove aspartic acid. Lead was removed from the filtrate with hydrogen sulphide and traces of bases precipitated with phosphotungstic acid in sulphuric acid solution. After removal of the excess of the latter reagents with barium hydroxide, the filtrate was made acid to Congo-red with sulphuric acid and concentrated to about 100 cc. The silver salt of β-hydroxyglutamic acid was then separated in the usual way and decomposed with hydrogen sulphide. The crude acid, as thus separated, was less pure than when prepared from caseinogen. It was purified by dissolving in cold 50 % methyl alcohol and precipitating with mercuric acetate in very feebly acid (acetic) solution. The precipitate, on decomposition with hydrogen sulphide and concentration at not over 50°, gave a very soluble syrup which

solidified in the desiccator and had all the properties of β-hydroxyglutamic acid prepared from caseinogen. The yield of acid was small but undoubtedly the losses were considerable. 100 g. of gliadin gave 2·4 g. of the acid, while 1·8 g. were obtained from 100 g. of glutenin. It is worth noting that the yields of the acid from caseinogen have been found to vary enormously. At times, not more than 2–3 % has been obtained in a reasonably pure state, while in other experiments as much as 10 % has been obtained. It is not unlikely that the acid, being a β-hydroxy-acid is not entirely stable to many of the reagents employed. The yields are generally highest when small amounts of protein are worked up at a time.

Acid from gliadin.

Analysis. 0·2112 g.; 0·2861 CO_2; 0·1100 H_2O.

	C	H
Calculated for $C_5H_9O_5N$...	36·8	5·52
Found	36·9	5·79

On heating the acid previously dried over phosphorus pentoxide to 110–115°, water was split off and the residue, containing 40·9 % of carbon and 5·3 % of hydrogen, was mainly made up of hydroxypyrrolidonecarboxylic acid, for it contained less than 1 % of nitrogen in the (NH_2) form as judged by van Slyke's method with nitrous acid.

The optical properties of the acid resembled those previously recorded. In aqueous solution $[\alpha]_D^{20°} = +1{\cdot}0°$ ($c = 5{\cdot}87$, $l = 2{\cdot}0$, $\alpha = +0{\cdot}11°$). In 20 % hydrochloric acid ($c = 1{\cdot}96$), $[\alpha]_D^{20°} = +14{\cdot}0°$.

Acid from glutenin. The acid obtained from glutenin resembled the preceding closely. It contained 37·0 % carbon and 5·8 % hydrogen. It showed the characteristic decomposition on heating at 110° but its concentrated aqueous solution showed no detectable rotation in 10 % aqueous solution and in 20 % hydrochloric acid it had $[\alpha]_D^{20°} = +6{\cdot}2°$. It appeared therefore as if partial racemisation had taken place in the process of preparation, for careful investigation failed to indicate the presence of any other acid. The strychnine and brucine salts were also prepared and analysed without disclosing any other cause for the lower rotations. It is not unlikely that racemisation may have been caused by the alkali used in the preparation of the glutenin.

VI. The fate of β-hydroxyglutamic acid in the diabetic organism.

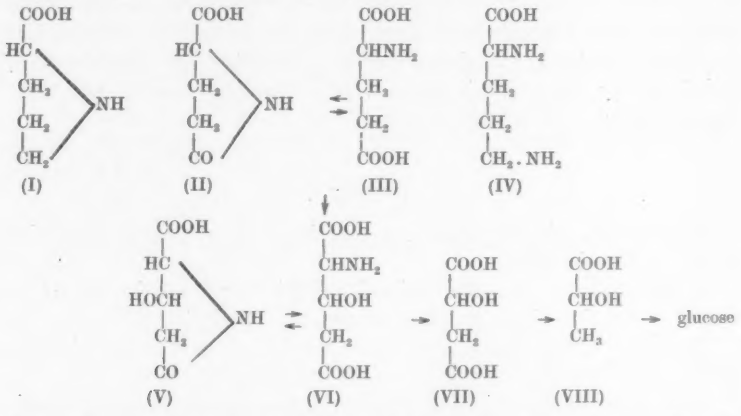
The fate of β-hydroxyglutamic acid in the diabetic animal presents certain questions of interest. Lusk has shown that when glutamic acid (III) is fed to a starving dog fully under the influence of phloridzin, about 65 % of the weight of glutamic acid given reappears in the urine as "extra glucose." The writer has shown that substantially the same thing is true for proline (I) and ornithine (IV), both of which, like glutamic acid, contain five carbon atoms. It appears as if three out of the five carbon atoms were concerned in glucose formation, and it is not unreasonable to regard it as likely that their catabolic paths are similar.

Now β-hydroxyglutamic acid has been found to yield glucose in the diabetic animal in precisely the same fashion as the previously mentioned acids. This fact appears to the writer to possess a certain speculative significance based on the probability of similarity in the metabolism of all of these acids. In the first place, if, as appears likely, β-hydroxyglutamic acid may arise in the body from glutamic acid, an example is furnished of "β-oxidation" such as is known to occur in the oxidation of fatty acids, but has not hitherto been observed with the amino-acids. Secondly, the possible conversion of β-hydroxyglutamic acid into malic acid (VII) by oxidation must be regarded as plausible for it involves a type of simple reaction often encountered in animal oxidation, and has, moreover, been effected by the writer *in vitro*. Malic acid is also stated to occur in sheep's sweat in significant quantities. Now Ringer, Frankel and Jonas [1913] have shown that malic acid readily goes over into glucose in the diabetic dog, 13 g. of the acid furnishing 8 g. of glucose. A similar conversion of malic acid into sugar has been observed in acorns by Bloor [1912]. It is therefore not unlikely that malic acid is a product of the intermediary metabolism of hydroxyglutamic acid and, in turn, of the other 5-carbon acids. In picturing the possible further steps in the conversion of malic acid into glucose, one cannot avoid considering favourably the idea that it may part with carbon dioxide with formation of lactic acid (VIII), for this is a reaction which is actually known to occur as the result of enzyme activity in the case of vegetable cells. This change is also observed in the ageing of wines and is apparently affected by *Micrococcus malolacticus* [cp. von der Heide, 1912]. The subsequent conversion of lactic acid into glucose involves no difficulty.

One other point may be referred to in connection with the rôle of β-hydroxyglutamic acid in metabolism. The conversion of proline into glucose obviously requires the opening of the pyrrole nucleus. The reversibility of many biological reactions lends significance to the extraordinarily easy conversion of β-hydroxyglutamic acid into hydroxypyrrolidonecarboxylic acid—and the less easy conversion of glutamic acid into α-pyrrolidonecarboxylic acid (II). Such changes may well be concerned with the synthesis of substances containing the pyrrole nucleus, and, moreover, the reduction of pyrrolidonecarboxylic acids to pyrrolidinecarboxylic acids is known to be feasible *in vitro* from Fischer's experiments.

It should be noted that Ringer, Frankel and Jonas have described experiments which they consider prove that glutamic acid is converted into succinic acid in the body, while they regard malic acid as an intermediate product in the metabolism of aspartic acid. They picture the malic acid giving hydracrylic acid and malonic acid, but curiously enough ignore the possibility of its giving lactic acid in spite of the fact that malonic acid gives little or no glucose in the diabetic animal. The writer is completely unable to follow the logic of Ringer's structural explanations of glucogenesis and cannot help feeling that too little distinction is made between possible metabolic paths and those actually known to occur.

The relationships in structure of the various five-carbon acids and their possible conversion into glucose *via* malic and lactic acids as previously outlined may be seen from the following scheme:



Experimental. Two experiments were made on the administration of β-hydroxyglutamic acid to a diabetic dog. The conditions of the experiments were those used previously by the writer [1913]. Phloridzin was administered to the starving dog by Coolen's method. 1 g. emulsified in 10 cc. of olive oil was given every day. The urine was collected in 18 hour periods and the bladder was carefully catheterised and washed out with sterile salt solution at the end of each period. The acid was given as the mono-sodium salt in aqueous solution by subcutaneous injection. The glucose was determined gravimetrically and checked by polarimetric readings. The excretion of "acetone bodies" was followed but showed no special change, hence the results are not recorded. The animal used was a terrier dog weighing about 8 kilograms.

Period	Hours	Total N	Glucose	*G*/*N* ratio	Substance
I	—	—	—	2·80	
II	18	4·21	13·53	3·22	
III	18	5·32	21·90	4·11	17·4 g. acid = 1·5 g. N
IV	18	5·10	15·74	3·08	
V	18	5·47	19·79	3·62	10 g. acid = 0·86 g. N

The "extra glucose" following the administration of the β-hydroxyglutamic acid may be calculated approximately using Lusk's method. The $\frac{\text{Glucose}}{\text{Nitrogen}}$ ratio in the blank periods averages 3·1. Assuming that the whole of the nitrogen of the amino-acid given is excreted in the course of the following 18 hour period, it is calculated that in the first experiment 17·4 g. of the acid gave rise to 10·06 g. of "extra glucose" while 10 g. of the acid gave 5·5 g. From the mean of the experiments it is concluded that β-hydroxyglutamic acid may furnish about 55–60 % of its weight of glucose in the diabetic dog.

According to the suggestion advanced that β-hydroxyglutamic acid may yield glucose in the diabetic animal *via* lactic acid, it may be calculated that the theoretical yield is 55 %, assuming one molecule of glucose to arise from two of lactic acid and two of hydroxyglutamic acid. The almost precise coincidence of the observed and calculated result must be regarded as partly accidental since the unavoidable experimental errors are considerable, but even allowing for them, the agreement is striking. The "protein sparing" effect of the injected amino-acid is very obvious.

Summary.

Considerable difficulty has been experienced in synthesising inactive β-hydroxyglutamic acid.

Bromination of the acetyl derivative of β-hydroxyglutaric anhydride and subsequent action of ammonia on the product did not yield the desired result.

Small amounts of the amino-acid were obtained on reducing α-isonitroso-acetonedicarboxylic ester but the results were unsatisfactory.

Only traces of the amino-acid were formed by the application of Strecker's reaction to malic semi-aldehyde.

The most satisfactory synthesis so far accomplished had as its starting point glutamic acid. The steps are as follows: glutamic acid → α-uramino-glutaric acid → hydantoin-propionic acid → hydantoin-β-bromopropionic acid → hydantoinacrylic acid → β-hydroxyglutamic acid.

It is noted that hydroxyaspartic acid has been synthesised from barium chloromalate.

The hitherto unknown semi-aldehyde of malic acid has been prepared from γ-diethoxyacetoacetic ester. Its *p*-nitrophenylosazone and that of tartronic semi-aldehyde are described.

The optical properties of β-hydroxyglutamic acid have been examined. The specific rotation of the free acid in 4 % aqueous solution is about + 0·8°, while a 2 % solution in 20 % HCl has a specific rotation of + 16·3°. The rotation of the potassium salt is practically undetectable, but the addition of uranium nitrate renders the solution strongly laevorotatory. On drying the free acid at 105° it loses water and is converted into hydroxypyrrolidonecarboxylic acid, which has a strong laevo-rotation in aqueous solution.

The strychnine and brucine salts of the following acids are described: *d*-β-hydroxyglutamic, *d*-glutamic, *l*-aspartic, and *l*-α-pyrrolidonecarboxylic.

β-Hydroxyglutamic acid has been isolated from gliadin and glutenin.

When administered to a diabetic (phloridzinised) dog β-hydroxyglutamic acid yields 55–60 % of its weight as glucose, apparently three of its five carbon atoms being concerned in glucose formation. In this it resembles glutamic acid, proline and ornithine, and it seems not unreasonable to regard the catabolic paths of all these acids as similar. The possibility of their conversion into glucose *via* malic and lactic acids is indicated.

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XXXVIII. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR: I. A MICROMETHOD FOR THE ESTIMATION OF ACETONE IN BLOOD, BASED ON THE IODOFORM METHOD.

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Scandinavian readers will find in my book [Widmark, 1917] a summary of the most important methods hitherto used for the estimation of the acetone concentration in blood and tissues. It will be found that none of the methods there referred to is entirely satisfactory or applicable to more extended measurements. Series of estimations upon living material cannot be obtained.

Of a method that is to be used both in the physiological laboratory and in clinical work the following demands, among others, should be made:

(*a*) It should not require more than about 100 mg. of blood. (*b*) The carrying out of the estimation should be simple, and the apparatus not too complicated or expensive. (*c*) The error should not exceed 10 %.

In the blood of diabetics with fairly pronounced acidosis the concentration of the total acetone fluctuates among values lying at about 0·20 per mille, *i.e.* in 100 mg. of blood there are about 20γ[1] total acetone. A micromethod should therefore have a mean error in the single estimation not greatly exceeding about $\pm 2\gamma$, which would therefore constitute an error of 10 % with a concentration of 0·20 per mille.

A detailed investigation of the iodoform method has shown that a modification of this renders possible an estimation of these small quantities of acetone with a mean error which remains approximately that just named. The carrying out of the estimation is simple and requires little practice. No more than 100 cmm. of blood is required, and the apparatus is not complicated.

We shall now describe the process, giving first the main outlines of the method and the carrying out of the estimation, and in a further section entering into a detailed examination of the method.

[1] We use the symbol γ for the quantity 0·001 mg., according to the proposal of the "Ausschuss für Einheiten und Formelgrössen" [1913].

A. Description of the micromethod.

1. *The method of obtaining the blood sample.*

The requisite amount of blood is obtained in the usual way by pricking with a stilette the ball of the finger or the ear-lobe. In disinfecting the skin care should be taken to remove all traces of alcohol and ether before the puncture is made. It is best merely to wash with soap and water. With a capillary pipette, graduated up to 100 cmm., the blood is sucked up as it issues from the wound. It is of great importance that the blood should not be subjected to evaporation, since in that case too low values may be obtained. The sucking up and measuring of the blood may be carried out most easily and most quickly by the aid of a precision regulator as constructed by Weichardt [1909][1]. The blood is brought into the flask of the distillation apparatus, in which there has previously been placed 10 cc. of 1 % phosphoric acid. The pipette is rinsed out four or five times with the phosphoric acid, so that every trace of blood passes down into the flask, which is immediately corked up.

The pipette should not be cleaned with alcohol and ether. The best method is to place it in the tube of a vacuum pump. After distilled water has been sucked through the pipette for a few moments, wipe the point. After a few minutes' further suction the pipette will be dry.

2. *The distillation.*

The distillation should be undertaken not more than two or three hours after the sample of blood has been secured. The shape of the distillation apparatus is shown by Fig. 1. From the distillation flask, the capacity of which is 100 cc., passes a detachable, twice-bent distillation tube. The tube is provided with a small drop-receiver and is blown out, immediately under the second angle, to a bulb of a few cc. capacity. It is drawn out at the end into a point the opening of which is about $\frac{1}{4}$ mm. across. A test-tube 150 mm. long and 15 mm. wide serves as receiver. The test-tube is enclosed by a cooler, the form of which is shown by the figure. The distillation tube reaches down into the test-tube and should end a couple of mm. above the bottom. The flask, distillation tube, and test-tube with accompanying cooler can be fixed in a wooden stand with elastic metal clamps in such a way that the test-tube can be loosened and withdrawn from the stand by a single movement.

In the test-tube, immediately before distillation, are placed 3 cc. of $N/2$ sodium hydroxide solution [natr. hydricum puriss. e natrio], and 2 cc. of $N/200$ iodine solution are added from a well-calibrated pipette (or in the estimation of greater quantities of acetone than 50γ, 2 cc. of $N/100$ iodine solution).

[1] A modification of this is sold by the A. B. Vetenskapliga Instrument (Scientific Instrument Company), Lund, Sweden.

Before distillation, the flask and the distillation tube should be boiled out for a few minutes with 1 % phosphoric acid. This is especially important if a cork connection is used between the tube and the flask.

In the distillation of pure water or phosphoric acid solution the strength of the titer of the alkaline iodine solution is diminished to some extent. In order to determine the titer two blind tests are carried out before the distilling

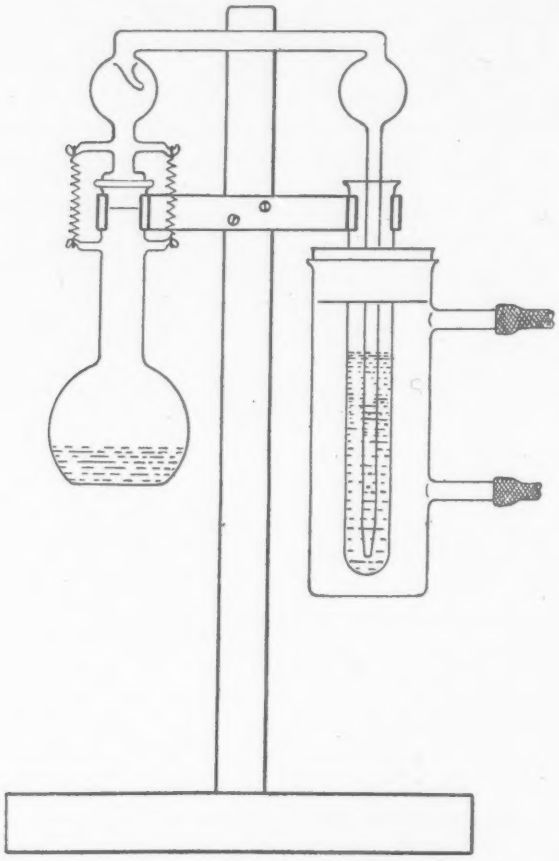

Fig. 1

of the blood sample, and one blind test afterwards. In these 10 cc. of 1 % phosphoric acid are placed in the flask, and the distillation is carried out as described below for the blood sample. The first blind test usually shows too great a consumption of iodine and should not be counted in the determination of the titer. The last will serve as a test by which it may be ascertained if any error has slipped in during the distillation of the blood sample.

The distillation of the blood sample is carried out as follows. The cork is removed from the flask and the distillation tube is attached. The apparatus is placed in the stand, and the condensing water turned on. The fluid is then boiled, the burner being held in the hand and the flame kept small. As soon as the steam has reached the contents of the test-tube a loud rattling will be heard. From this moment the boiling is continued for 100 seconds, the heat being moderated by raising and lowering the burner, so that the boiling remains even and the blood does not froth up into the distillation tube. A test of the evenness of the boiling exists in the rattling noise, which ought not to cease. When the 100 seconds are up, the heat is increased a little and at the same time the test-tube is loosened from its clamp, so that the point of the distillation tube comes above the surface of the fluid. No fluid must be allowed to remain clinging to the point; any that does so remain can be blown down into the test-tube by heating the flask for a moment. When the test-tube has been further cooled down for a few minutes, the condensing water is shut off and the test-tube is taken out of the cooler. We may now proceed to the next distillation; 10 distillations can be carried out in an hour. A precipitate appears only on the distillation of quantities of acetone exceeding 50γ, the iodoform being to a certain extent soluble in water.

3. *The titration.*

When the tube has stood for at least three minutes, 3·5 cc. of $N/2$ sulphuric acid are added from a burette. In every tube is placed a thin glass rod, the lower end of which is flattened out into the form of a disc, in order to facilitate the stirring of the fluid. The solution is stirred by moving this glass rod a few times up and down. A couple of drops of a 1 % starch solution are now added with a pipette, and titration is carried out with $N/200$ sodium thiosulphate solution (or $N/100$ if $N/100$ iodine solution be used). In the titration we use a burette (Bang's burette for the estimation of blood sugar) graduated in 0·02 cc. Thiosulphate is added drop by drop as the fluid is stirred with the glass rod, until the blue colour almost vanishes. Towards the end of the titration only fractions of a drop are added. The titration is continued until every trace of colour has vanished; the easiest way to find out when this is the case is to look at the fluid through the mouth of the test-tube. With a little practice the change can be determined to somewhat less than half a drop. Care must be taken that no drops of thiosulphate remain clinging to the upper parts of the test-tube. They can be brought down with the glass rod.

The blind test enables us to determine the titer of the iodine solution. The amount of acetone is calculated from the difference between the quantities of thiosulphate used in the blind test and in the blood test. 0·02 cc. of $N/200$ thiosulphate corresponds to 0·968γ acetone.

B. Detailed examination of the method.

For the testing of the method pure acetone was used, prepared in the following way from the bisulphite compound [see Plimmer, 1915]. To 125 cc. of crude acetone were added 70 g. of sodium bisulphite in saturated solution. The mixture was well shaken in a closed flask and, after being left to stand for some time, the liquid was drawn off by means of a suction filter and the precipitate dried between filter-paper. It was then placed in a distillation flask and decomposed with 40 g. of soda. The mixture was subjected to fractional distillation, and the distillate was dried with calcium chloride and subjected to a fresh distillation. The fraction that passed over at a temperature of between 56 and 57° was used in the experiments.

The pure acetone boiled at a temperature of + 56·8°, and had a sp. g. of 0·798 at 15°. Its reaction was neutral and it showed no aldehyde reaction (equal volumes of 10 % silver nitrate and sodium hydroxide were mixed together, ammonia was added drop by drop until the precipitate was dissolved, and a couple of drops of acetone were then added. No mirror was formed).

A solution prepared from 1·462 g. acetone in 1 litre of water showed the following concentration, when titrated by Messinger's method:

$$1{\cdot}465 \pm 0{\cdot}0023 \text{ per mille (6 estimations).}$$

The acetone may therefore be regarded as sufficiently pure for the testing of the micromethod.

In order to render possible the preparation of an acetone solution of accurately known concentration and to avoid any loss through evaporation in the measuring of the pure acetone, the following method of procedure was adopted.

A number of small bulbs of 1–2 cc. capacity were blown out from narrow glass tubes and the point of each bulb was drawn out into a capillary tube. The bulb, after being weighed, was filled with the pure acetone by gently heating it and then dipping the capillary into the acetone. As cooling took place the bulb was filled with the fluid. The capillary was then melted off, and the filled bulb weighed again.

In the preparation of dilute acetone solutions a 1 litre volumetric flask was half filled with water, and a bulb was dropped into the water and broken with a glass rod. The flask was then filled up to the mark. Finally there was further added a small quantity of water corresponding to the volume of the glass of the bulb, the sp. g. of which was assumed to be 2·5.

1. *Measurement of the blood sample.*

The greatest risk of error in the measurement of the blood sample lies in the fact that the acetone easily volatilises. Opportunity for this is found in the time which elapses from the moment when the blood comes out of the puncture until it is sucked up into the pipette. In the pipette the danger of evaporation is extremely small.

Generally I have proceeded in the following way: 4–5 drops of blood from the puncture were allowed to drop on to a clean watch-glass, from which the blood was quickly sucked up into the pipette. The time required from the falling of the first drop of blood upon the glass until the requisite quantity had been sucked up into the pipette usually ran to 15–20 seconds. During this period the evaporation is so small that it is not noticeable with the micromethod.

This method has however been sharply criticised, and it must be granted that the method is associated with a certain risk for persons without practice. I recommend therefore that the blood be not allowed to drop upon a watch-glass. Instead it may be sucked up directly by placing the point of the pipette over the puncture and sucking up the blood as it drips out. This can be done very easily and conveniently by means of the Weichardt regulator mentioned above. With a blood sample from the ear of the rabbit it is best to let an assistant hold the animal's ear stretched out upon a square of glass. This will give the necessary support and firmness for the sucking up of the blood as it drips out.

That the taking of the sample in this way gives a perfectly satisfactory result is shown by the following experiments.

Experiment 1. A quantity of acetone was subcutaneously injected into a rabbit. Three hours later seven blood samples were taken; nos. 1, 3, 5 and 7 by means of the pipette, nos. 2, 4 and 6 by direct dropping of the blood from the ear vein into weighed flasks. Room temp. 17°.

The samples taken with the pipette gave the following values:

1	0·392 °/₀₀
3	0·392
5	0·390
7	0·387
Average	0·390

The weighed samples gave the following results:

2	123·2 mg.	blood	contained	46·5γ	acetone	=0·377 °/₀₀
4	66·6	„	„	24·4γ	„	=0·366
6	124·0	„	„	44·0γ	„	=0·355
				Average		0·366

Experiment 2. Similar experiment with a greater quantity of acetone. Room temp. 16·5°.

With the pipette:

2	0·629 °/₀₀
4	0·624
6	0·615
8	0·620
Average	0·622

By dropping directly into weighed flasks:

1	68·8 mg.	blood	contained	43·3γ	acetone	=0·629 °/₀₀
3	84·4	„	„	50·3γ	„	=0·596
5	113·4	„	„	67·8γ	„	=0·598
7	106·0	„	„	67·3γ	„	=0·635
9	80·2	„	„	46·9γ	„	=0·585
				Average		0·609

By weighing, somewhat lower values are found than when the pipette is used, this being evidently due to the fact that in the former case the blood is measured in mg., in the latter in cmm. If we assume the sp. g. of the blood to be 1·05 and calculate the weighed samples according to this, we find the average in Exp. 1 to be 0·384 ‰ and in Exp. 2 0·639 ‰, the former value a little lower, the latter a little higher, than the average of the samples measured with the pipette.

The measurement with the pipette is simpler and easier than weighing and is therefore to be preferred.

The risk of evaporation however is sufficiently great to put out of the question a sucking up of the blood and weighing with a torsion balance in the same way as in the blood-sugar test according to Bang. On the other hand it is possible that this method may be very well suited to an estimation of aceto-acetic acid. For this purpose the blood may be sucked up on to thin filter-paper and weighed, and the free acetone may be allowed to evaporate either by letting the filter-paper hang for some time under ordinary atmospheric pressure, or by enclosing it for a few minutes in a flask which is then evacuated by means of a water suction pump. This process is at present being worked out, but the details are not ready for publication. If Shaffer's [1911] method for converting β-hydroxybutyric acid into acetone and then estimating the acetone should prove to be practicable, this micromethod could probably also be used with advantage for a micro-estimation of β-hydroxybutyric acid in the blood.

2. *The distillation.*

The distillation of the blood sample presents no noteworthy difficulties. The construction of the distillation apparatus may vary in different ways. An indispensable condition however is that the steam must not be allowed to come into contact with either rubber stoppers or tubes. It seems, in fact, as if the steam possessed the power of carrying along with it substances which unite with iodine. The consequence of this is that the estimations become too high: the error may often amount to several tenths per cent of the real value. Cork may be used, but it must be well boiled previously. It is safest to use an apparatus entirely of glass, with distillation tube ground in. The disadvantage of such an apparatus however is that it is impossible without great expense to get a considerable number of distillation flasks of the right size to fit into one and the same distillation tube. This fact makes it difficult to undertake series of estimations.

The advantage of the apparatus previously described is that it is particularly simple both in construction and management. I have generally used cork connections between distillation tube and flask. This was the case in all the estimations in this part of the work. Care should be taken that the open end of the distillation tube is not too large, and that the tube does not end more than 4 mm. above the bottom of the test-tube.

The fact that I allow the acetone distilled over to be absorbed without previous cooling by the alkaline iodine solution, which in the course of the distillation has been heated to a considerable extent, might possibly lead to the supposition that great loss would be unavoidable, or at least would very easily arise. Practical experience however shows—as appears quite clearly in the chapter on the precision of the method—that the risk of a loss of acetone is very small. Moreover

there is also some theoretical support for the view that the absorption of the acetone ought not to be impaired by a gentle heating of the alkaline iodine solution. The decisive tests for the retention of the acetone in the iodine solution are in the first place its vapour-pressure in the solution, and secondly the velocity of the reaction in the formation of iodoform. With a rise of temperature both these factors are intensified, but they act in opposite directions. The increased vapour-pressure facilitates the evaporation of the acetone, the increased speed of the reaction renders it possible for greater quantities of acetone to be bound as iodoform within the unit of time. The loss of acetone will depend on the question which of these two factors is increased most with a given rise of temperature. According to Regnault [1862] the tension of the acetone is increased from 179·6 mm. Hg at 20° to 420·2 mm. at 40°, a rise therefore in the proportion of approximately 1 : 2. The partial pressure in an aqueous solution of 1 % is increased, according to my own measurements, in approximately the same proportion with a rise in temperature from 20 to 40°. If we assume that the velocity of the reaction in the formation of iodoform follows van't Hoff's law, the velocity for a rise in temperature of 20° should be increased in the proportion of 1 : 4. Hence it follows that the retention of the acetone in the alkaline iodine solution is facilitated to an appreciable extent by heating. In this connection however another circumstance of great importance is to be mentioned. It is an established fact that when iodine is added to alkali the resulting solution does not remain unchanged if left to stand. The hypoiodite formed at the beginning passes over by degrees into iodate:

$$3KOI = KIO_3 + 2KI.$$

The iodate does not form iodoform with acetone. The formation of iodate proceeds slowly in cold solutions, but almost instantaneously in warm. This fact would therefore seem to argue that the binding of the acetone is rendered more difficult by the heating of the solution, and that an error in the estimation might arise owing to the fact that the quantity of hypoiodite necessary for the formation of iodoform is not forthcoming. In my own experiments I have never been able to find that the circumstance just mentioned plays any part in the estimations, although I have often made use of the supply of iodine in the alkaline iodine solution up to 70–80 % (see *e.g.* experiment 9). Possibly the case is somewhat otherwise with these extremely dilute solutions than with the more concentrated.

Determination of the most suitable time of distillation.

Experiment 3. In the distillation flask are placed 10 cc. of 1 % phosphoric acid and 0·1 cc. of 0·732 °/₀₀ acetone solution. The flask therefore contains 73·2γ acetone. The alkaline iodine solution is introduced into the test-tube in the usual way, 2 cc. of $N/200$ iodine solution being used. The experiment is repeated eight times, with variations in the time of distillation, the latter being reckoned from the point at which the rattling sound begins.

Table I.

Time of distillation in min.	Acetone in γ
$\frac{1}{4}$	55·1
$\frac{1}{2}$	64·5
$\frac{3}{4}$	68·0
1	72·8
$1\frac{1}{4}$	74·3
$1\frac{1}{2}$	72·8
$1\frac{3}{4}$	74·8
2	73·8

The experiment shows that after 1 minute practically all the acetone has already been distilled over.

Experiment 4. A similar experiment, with the use of 168·6γ acetone in the flask and 2 cc. of $N/100$ iodine solution.

Table II.

Time of distillation in min.	Acetone in γ
½	143·9
¾	161·4
1	169·1
1½	170·8
2	168·1
2½	168·6
2¾	173·0

Here too the acetone has passed over quantitatively after 1 minute.

The experiment shows that a time of 90 seconds is sufficient for the distillation of pure solutions. In distilling blood samples it is necessary, on account of the frothing, to boil somewhat more carefully. I recommend therefore a somewhat longer time—100 seconds—for the boiling.

The dilution of the blood sample with 10 *cc. of* 1 % *phosphoric acid* is designed partly to check the frothing in the distillation, and partly to decompose the aceto-acetic acid. The dilution is carried out in the proportion of 1 : 100. In the distillation about 4 cc. pass over, so that afterwards the dilution is in the proportion of about 1 : 60. This dilution is more than ten times as great as that recommended by Embden for the urine distillation. There can therefore be no reason to suppose that any disturbing iodine-binding substances will, through the concentration due to the distilling process, be made to pass over into the distillate. A dilution of the sample with water while distillation is in progress is superfluous.

3. *The titration.*

After the distillation the tube should stand for 1 minute in the cooler. After a further period of 3 minutes the formation of iodoform is finished. This is shown by the two following experiments.

Experiment 5. To each of six tubes containing 3 cc. of sodium hydroxide and 2 cc. of $N/200$ iodine solution is added 1 cc. of an acetone solution containing 47·4γ per cc. The first tube is acidified immediately, the second after 1 minute, the third after 2 minutes, and so on. The results of the titration are shown by the following table.

Table III.

Time in min.	Acetone in γ
0	22·5
1	36·3
2	45·5
3	47·4
5	47·6
20	47·4

The first column shows the time that elapsed between the adding of acetone to the alkaline iodine solution and the addition of acid.

In this experiment therefore the iodine is already bound after 3 minutes.

Experiment 6. To each of four flasks, containing 10 cc. of 1 % phosphoric acid, 0·1 cc. of acetone solution containing 53·2γ acetone was added. Distillation was then carried out in the usual way. The test-tubes were allowed to stand in the cooler for 1 minute. The first tube was acidified immediately after this, the second after 2 minutes, and so on.

Table IV.

Time in min.	Acetone in γ
0	52·6
2	53·3
15	52·2
20	52·2

The experiment shows that the mixture may be acidified 3 minutes after the tube is taken out of the cooler.

After the acidification and when the solution has been sufficiently stirred with the glass rod, starch may be added and titration carried out.

Experiment 7. To each of five tubes containing alkaline iodine solution was added 1 cc. of an acetone solution containing 68·8γ acetone per cc. When the tubes had stood for 5 minutes they were acidified with sulphuric acid. The contents of the first tube were titrated immediately, those of the second after half a minute, and so on.

Table V.

Time in min.	Acetone in γ
0	67·4
½	67·4
1	67·4
10	67·9
30	66·9

The first column shows the time that elapsed between the addition of sulphuric acid and the titration.

It is of a certain importance that the titration should be carried out in the test-tubes, partly because the loss of colour can be more clearly observed there than in flasks and beakers, and also because the risk of evaporation of the iodine is then quite inconsiderable. Titrations in flasks and beakers always require a somewhat smaller consumption of thiosulphate than titrations in test-tubes. An iodine solution which, when titrated in a test-tube, used up 1·98 cc. of $N/200$ thiosulphate, used in a 50 cc. flask 1·95 cc., and in a beaker, after 5 minutes' gentle stirring, only 1·40 cc.

The diminution which appears on distillation in the titer of the iodine generally reaches a degree corresponding to 0·03–0·05 cc. of $N/200$ iodine solution. Probably this diminution is due to several causes.

In the text-books of quantitative analysis stress is laid upon the fact that the quantity of iodine demanded for the production of the blue colour will vary considerably, according to the concentration of the iodide present. Care must therefore be taken that the analyses are carried out as far as possible in equal volumes of fluid. With the use of $N/10$ iodine solution however the error becomes so small that it may as a rule be neglected. If $N/100$ iodine solution be used the error may become very great. Treadwell [1913] therefore carries out the following experiment.

To different quantities of water were added 1·5 cc. of starch solution, and $N/100$ iodine solution until the first perceptible blue coloration appeared.

Water in cc.	$N/100$ iodine solution in cc.
50	0·15
100	0·30
150	0·47
200	0·64

When, on the other hand, there was added to the solutions 1 g. of potassium iodide, the consumption of iodine was reduced to about 0·04 cc. up to a volume of 200 cc., which required 0·14 cc. of iodine solution.

In the micromethod the volume of fluid is about 12 cc. and varies within very narrow limits. I have not been able to find that the result of the titration is changed by adding potassium iodide immediately before titrating. Probably sufficient quantities are already present.

The reason for the change in the titer of the iodine solution is probably to be sought in part in another direction. The consumption of iodine becomes greater if, in distilling, the boiling is prolonged beyond the time prescribed. It is greater when a rubber stopper is used as connection between flask and distillation tube than with the use of cork, and is least of all with only glass connections. Finally we find a diminution in the titer of the iodine as soon as we pass 100 cc. of air free of dust through the alkaline iodine solution.

The determination of the titer can however be carried out with perfectly satisfactory accuracy by means of the two blind tests, provided only that the titer of the thiosulphate is quite correct. The result shows that the titer remains sufficiently constant even during very long series of experiments. It is however of great importance for the obtaining of good results that the purest possible chemicals should be used. Only the sodium hydroxide prepared from the metal is of perfectly satisfactory purity. The other preparations often give, even with the test-sample without distillation, too great a consumption of iodine. Ordinary distilled water (according to recent investigations in connection with the salvarsan treatment) often contains important quantities of organic substances which may of course bind iodine. It has been proved also that newly-distilled water gives considerably more even results than ordinary distilled water. I have therefore, in the preparation of all the solutions required by the method, myself distilled the water in a glass distillation apparatus[1].

4. *The standard solutions.*

The preparation and conservation of these is effected by means of the methods that will be found in most of the text-books of analytical chemistry[2].

[1] A very convenient apparatus of this kind has been constructed by Hamner and may be obtained from Grave, Stockholm.

[2] Naturally there can here be no question of the preparation of $N/200$ iodine solution according to Bang by the addition of titrated acid to an excess of iodate and potassium iodide. For when the solution after the distillation is mixed with excess of acid, there is liberated a further quantity of iodine corresponding to the excess of acid.

I have found it safest and most convenient to prepare every day from well-determined $N/10$ iodine and thiosulphate solutions new solutions of $N/100$ and $N/200$ respectively. We have a control of these in the blind tests. The standard solutions should be tested at least once a week. This is most easily done by first testing the thiosulphate solution with bichromate and potassium iodide in a solution acidified with sulphuric acid.

5. *Accuracy of the method.*

The two following experiments are designed to illustrate the accuracy of the method in the estimation of the amount of acetone in pure solutions before and after the distillation.

Experiment 8. In 12 test-tubes were measured out 3 cc. of $N/2$ sodium hydroxide + 2 cc. of $N/200$ iodine solution + 3 cc. of freshly distilled water. From an acetone solution containing 73·1 γ acetone per cc. 0·1 cc. was measured off (with a 1 cc. measuring pipette) in the first tube, 0·2 cc. in the second, and so on; 1 cc. in the tenth tube therefore. The eleventh and twelfth tubes served as blind tests for the determination of the titer of the iodine. After 5 minutes 3·5 cc. of $N/2$ sulphuric acid was added to each of the tubes. After stirring with a glass rod titration was immediately carried out. The following table and Fig. 2 show the results of the titration.

Table VI.

	Calculated	Found (γ)
1	7·3	10·0
2	14·6	15·4
3	21·9	20·8
4	29·2	30·6
5	36·6	37·8
6	43·9	44·9
7	51·2	51·3
8	58·5	61·0
9	65·8	66·8
10	73·1	73·9

The table shows the titration results in the estimation of pure acetone solutions without previous distillation. The second column gives the calculated quantity of acetone, the third column the quantity found by the estimation.

Fig. 2 illustrates this experiment.

Experiment 9. In each of 13 flasks 10 cc. of 1 % phosphoric acid was measured off. Into ten of these flasks rising quantities (from 0·1–1·0 cc.) of an acetone solution containing 73·1 γ acetone per cc. were measured off with a 1 cc. measuring pipette. Distillation was then carried out in the usual way, with two blind tests before, and one blind test after, the acetone tests. In the test-tubes 3 cc. of $N/2$ sodium hydroxide + 2 cc. of $N/200$ iodine solution. The acetone values when plotted as in Fig. 2 fall on a straight line.

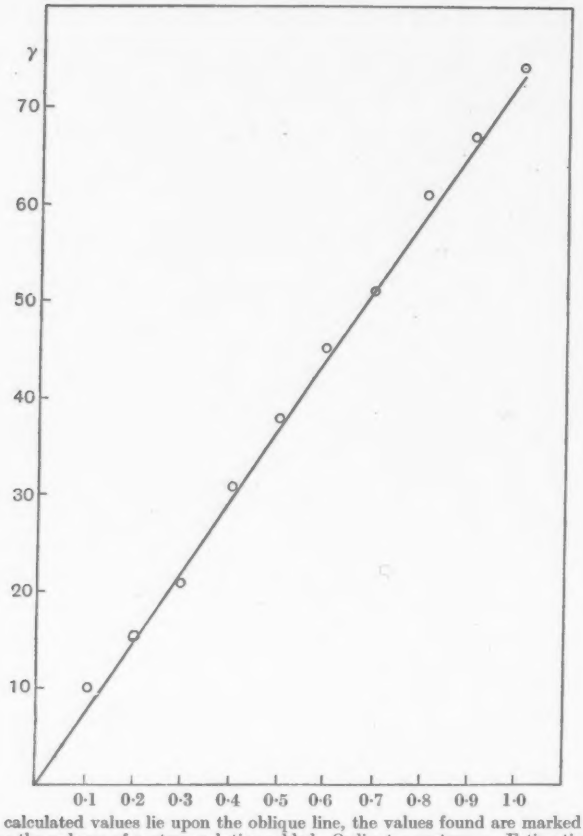

Fig. 2. The calculated values lie upon the oblique line, the values found are marked by circles. Abscissa: the volume of acetone solution added. Ordinate: acetone γ. Estimations without distillation according to experiment (8).

Table VII shows the titration results in an estimation of pure acetone solutions after distillation. Arrangement as in the previous table.

Table VII.

	Calculated	Found (γ)
1	7·3	11·1
2	14·6	16·0
3	21·9	21·8
4	29·2	29·5
5	36·6	38·0
6	43·9	44·0
7	51·2	50·8
8	58·5	57·1
9	65·8	66·2
10	73·1	73·0

The mean error of the single estimation in an estimation of pure acetone solutions appears from the following series.

Experiment 10. Ten estimations were carried out on a pure acetone solution containing 7·32γ per 0·1 cc. The acetone was measured out with a 0·1 cc. pipette. Distillation as usual. In the test-tubes 3 cc. of hydroxide + 2 cc. of $N/200$ iodine solution. The following values were obtained[1]:

Acetone in γ: 9·7, 7·7, 7·3, 7·1, 7·3, 7·5, 7·3, 7·3, 8·0, 6·8.

Average: 7·6, $E = \pm$ 0·805, $e = \pm$ 0·255.

Experiment 11. Ten estimations on an acetone solution containing 57·1γ per 0·1 cc. Estimations carried out as before.

Acetone in γ: 56·7, 56·2, 56·6, 56·7, 56·7, 56·2, 56·0, 57·9, 53·7, 55·7.

Average: 56·24, $E = \pm$ 1·071, $e = \pm$ 0·339.

From these two experiments it follows that the mean error of the separate estimation hardly exceeds 1γ. The absolute value of the error is about the same, independently of the amount of the acetone. In the first series the error expressed as a percentage of the mean is 10·5 %, and in the second series 1·9 %.

In the first series the mean is somewhat higher than the calculated value, in the second series somewhat lower. I have not been able to find—provided that sufficient attention is paid to the estimation of the consumption of iodine in the blind experiment—that the method has any appreciable constant error. In general, however, the values are a little too low (about 1γ). This is especially the case if a distillation tube is used having a point with a wider opening than that mentioned in the description of the apparatus. Under such circumstances it may happen that in the distillation the air which passes out before boiling is set up is not completely freed from its acetone in passing through the alkaline solution. Further it is necessary to provide carefully that the glass connection shall close quite tightly. It is advisable to damp the ground glass with a drop of distilled water when the glass tube is fitted into the flask. If a cork connection is used the same cork should not be used too long, but should frequently be replaced by a new one.

It is evident that the method is not suitable for estimations of the acetone percentage in the normal blood. For this 0·1 cc. of blood is too small a quantity. The estimation of the concentration in normal blood falls entirely within the limits of error of the method. In an estimation of normal blood we therefore regularly get titration results which are identical with those of the blind test, or at most show 0·01–0·02 cc. less $N/200$ thiosulphate consumption than these.

The following experiment shows the mean error of the separate estimation in a determination of the amount of acetone in calves' blood, to which acetone was added:

Calculated γ	79·6	39·8	31·8	19·9	15·9	8·0
Found γ	77·1	39·9	32·7	18·9	14·4	8·0
	77·1	39·4	30·3	19·4	15·4	8·0
	77·0	41·3	30·7	19·6	15·9	7·5
	76·7	39·4	30·3	19·1	16·8	7·5
	73·8	40·4	32·2	19·8	16·1	7·7
Average	76·3	40·1	31·2	19·4	15·7	7·7

[1] By E, here and in the following experiment, is meant the mean error of the single estimation; by e the mean error of the mean.

It is my impression that as a rule we are rather more likely to get too low values than too high. In order to illustrate the agreement between the two-fold estimations in the determination of the total acetone concentration in the blood of *diabetics* with acidosis, I give the following report of all the estimations in a case. This series is intended to demonstrate the clinical practicability of the micromethod. The samples of blood were taken by the Hospital laboratory nurse, the distillation was performed by my own laboratory assistant, and finally I myself carried out the titrations.

Table VIII.

	Acetone ‰	
Date	I.	II.
24. i.	0·302	0·321
25. i.	0·334	0·334
26. i.	0·310	0·314
27. i.	0·189	0·184
28. i.	0·114	0·111
29. i.	0·080	0·082
30. i.	0·116	0·097
31. i.	0·264	0·276
1. ii.	0·165	0·160
2. ii.	0·172	0·174
3. ii.	0·126	0·102
4. ii.	0·109	0·114
5. ii.	0·097	0·097
6. ii.	0·106	0·087
7. ii.	0·106	0·087
8. ii.	0·068	0·073
9. ii.	0·204	0·216
10. ii.	0·174	0·174
11. ii.	0·135	0·135
12. ii.	0·094	0·082

In order to show that in the short period of distillation there is time for all the aceto-acetic acid present to be decomposed, a few analyses of the total acetone concentration in urine have been made. In these the total acetone has been estimated by the method of Embden and Schliep and the micromethod simultaneously. The results are shown by the following table.

Table IX.

Subject	Macromethod	Micromethod	
A. S.	0·198	0·208 0·203	0·206
A. S.	0·397	0·392 0·375	0·385
Mrs L.	1·083		1·060
H. S.	0·329	0·314 0·315	0·315
H. S.	0·295	0·305 0·300	0·303

The experiments show that with distillation by the micromethod aceto-acetic acid is decomposed to the same extent as when the macromethod is used.

As we saw in regard to the blood estimations, so also it is impossible to estimate the normal acetone percentage in the urine with any claim to accuracy by the micromethod. As an average figure for the quantity of total acetone present in normal urine may be given approximately 20 mg. per daily quantity of urine. Assuming a daily quantity of 1500 cc. the quantity of acetone present in 0·1 cc. is therefore only a little more than 1γ. The estimation of this quantity falls entirely within the limits of error of the method, which fact was also shown by series of estimations.

Finally it should be emphasised that we do not yet know with perfect certainty whether the values obtained by estimating by means of the iodoform process correspond in every respect to the acetone concentration in question. The possibility is still open that besides acetone other volatile iodoform-producing substances pass over into the distillate in an estimation of the blood and urine of diabetics. Such substances may in general be characterised as having the group CH_3C united with oxygen.

The presence of *ethyl alcohol* in the blood of diabetics demands special attention for the reason that these patients are often given a certain amount of wine in their diet.

However the presence of ethyl alcohol in the blood has no effect upon the results of titration.

Experiment 12. Subject: the author. At 2.15 p.m. on April 15, 1917, 50 cc. of whisky were taken $2\frac{1}{2}$ hours after a meal. This quantity contains about 25 cc. of absolute alcohol (and about 9 mg. of aldehyde, reckoned as acetaldehyde). Before the spirit was drunk two blood samples were taken, and a double sample was also taken 25 and 45 minutes respectively after drinking.

Iodine solution consumed in the blind tests: 1·930 and 1·935 cc. $N/200$ thiosulphate. The iodine solution consumed in the blood tests corresponded to the following quantities of $N/200$ thiosulphate:

Table X.

Blood test.		
Before the taking of the alcohol	1·940	1·940
At 2.40 p.m. (25 min. after taking)	1·940	1·940
At 3.0 p.m. (45 min. after taking)	1·940	1·930

In this experiment the alcohol concentration in the blood during the time taken for the last two tests certainly exceeded 0·5 $^0/_{00}$ notwithstanding the fact that no change in the titer of the iodine can be observed in the tests.

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XXXIX. STUDIES ON THE CYCLOCLASTIC POWER OF BACTERIA.

PART I. A QUANTITATIVE STUDY OF THE AEROBIC DECOMPOSITION OF HISTIDINE BY BACTERIA.

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REPORT TO THE MEDICAL RESEARCH COMMITTEE.

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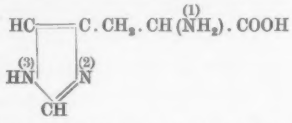
The investigation of the products formed during the decomposition of histidine by micro-organisms has led to the isolation of a few compounds of biochemical interest. Ackermann [1910] obtained a 42 % yield of histamine (β-iminazolylethylamine) as a result of the growth of mixed putrefactive bacteria on solutions containing histidine. This discovery became of intense interest when Barger and Dale [1910] showed the remarkable physiological activity of this amine, and identified it as one of the active principles of ergot. More recently, Mellanby and Twort [1912] have observed the formation of this amine by pure cultures of an unnamed bacillus which they isolated from the alimentary canal. Berthelot and Bertrand [1912] also observed that a similar decarboxylation of histidine, resulting in the formation of histamine, is readily accomplished by a bacillus to which they gave the name of *Bacillus aminophilus intestinalis*. In fact, the bacterial method of preparation of histamine is used industrially by Hoffmann, La Roche and Co.

It is stated by Windaus and Opitz [1911] that F. Ehrlich obtained β-iminazolylethyl alcohol by the action of yeast on histidine, but I have failed to find that Ehrlich has published his results.

Of the acids which might arise from histidine, by the action of micro-organisms, only two have been isolated. In the paper previously referred to, Ackermann [1910] obtained, in addition to histamine, a very small amount of β-iminazolylpropionic acid. The present writer [1917], working with pure cultures of bacteria of the coli-typhosus group, obtained the unsaturated acid, urocanic acid (β-iminazolylacrylic acid), from histidine. The yield in some cases—in particular with *B. paratyphosus A*—was of the order of 60 % of the histidine employed. Later, in extending this work to organisms of other groups, it was found—more particularly with *B. pyocyaneus*—that, although a considerable amount of ammonia was produced and hence at any rate part of the histidine was decomposed, it was impossible to isolate any iminazole

derivatives, except unchanged histidine, from the culture media. For this reason, it was decided to investigate quantitatively the breakdown of the histidine molecule by bacteria. The results of this investigation are given in this paper.

During this work, the fate of the nitrogen contained in the histidine molecule was the only question studied, since, as will be immediately shown, this enables one to form a good idea not only of the fate of the side chain, but of the iminazole nucleus as well. A consideration of the formula for histidine



indicates that the N (numbered (1)) in the side chain may be easily differentiated from the N (numbered (2) and (3)) in the ring. Van Slyke [1911, 1] has indeed shown that, when histidine is treated with nitrous acid—as in his method for the estimation of amino acid nitrogen—only one-third of the total nitrogen in the molecule is liberated as nitrogen, *i.e.* the amino group in the side chain.

Using this as the basis of the method employed, the following estimations were performed at stated intervals on media containing histidine dissolved in Ringer's solution, and sown with the organism under investigation.

A. Total Nitrogen (by Kjeldahl) gives the sum of the three nitrogen atoms ((1), (2) and (3)) whether present, as at the commencement of the experiment, as histidine, or, as in the later stages, as products of the breakdown of histidine.

B. Amino Nitrogen (by van Slyke [1912]) gives the nitrogen (1) contained in the amino group of the side chain, thus providing a measure of the rate of breakdown of the amino group.

C. Ammonia Nitrogen gives the whole of the nitrogen, whether of the side chain or nucleus, which is metabolised by the bacteria into ammonia.

By simple calculations the following values can also be determined:

D. Total Non-amino Nitrogen (other than ammonia nitrogen). The difference between the total nitrogen (A) and the sum of the amino nitrogen and ammonia nitrogen ($B + C$) gives one the *total* non-amino nitrogen present at any stage,

$$i.e.\ A - (B + C) = D.$$

In other words, D gives one a measure of the rate of degradation into ammonia of the nitrogen contained in the nucleus.

E. Non-amino Nitrogen (other than ammonia nitrogen and the non-amino nitrogen present as unchanged histidine).

Since the ratio of non-amino nitrogen to amino nitrogen in histidine is 2 : 1, the amount of non-amino nitrogen present at any stage as unchanged histidine will be double the amount of amino nitrogen, *i.e.* $2B$. Thus

$$E = D - 2B.$$

In other words, *E* gives a measure of the rate of production from histidine of compounds (other than ammonia) containing non-amino nitrogen.

It will be noted that no account is taken of the nitrogen which may be used for synthesis, *e.g.* in the formation of bacterial protein. It is, however, very probable, as is shown from results obtained with *B. proteus*, that the amount of nitrogen used for synthesis by these bacteria is relatively negligible (with the possible exception of *B. pyocyaneus*) and does not vitiate the general conclusions drawn from these experiments.

Experimental.

In all cases, the culture solution was contained in a two litre flask, fitted with a rubber stopper carrying two glass tubes. One of these, bent at an angle of about 60°, was fitted at one end with a plug of cotton wool about three inches long, and the other end was inserted, to a distance of about an inch, through the rubber stopper. The other tube, bent at the same angle, was fitted at one end with a piece of rubber tubing, carrying a small glass tube drawn out to a sealed capillary point. The other end of the tube was inserted through the rubber stopper, and terminated about $\frac{1}{4}$ inch from the bottom of the flask. The apparatus thus closely resembled an ordinary wash bottle, except that the tube carrying the cotton wool plug was bent downwards, to lessen the risk of contamination.

The following solution was then introduced into the flask, and the whole was autoclaved at 120° for an hour.

Distilled water	1550 c.c.
Sodium chloride	16·2 g.
Potassium chloride	0·756 g.
Calcium chloride	0·432 g.
Normal caustic soda	23·2 c.c.

(to neutralise the HCl of the histidine hydrochloride).

In a separate 300 c.c. conical flask, 4·86 g. of pure histidine monohydrochloride was dissolved in 250 c.c. of water, and autoclaved. This separate autoclaving was preferred, as histidine undergoes undesirable changes to a far less extent in acid solution (as the hydrochloride) than in alkaline solution (as the free base).

The contents of the two flasks, after mixing, thus gave a 0·2 % solution of histidine in Ringer's solution, this being the medium previously used [Raistrick, 1917]. In order to introduce the solution of the histidine hydrochloride into the large flask, the following procedure was used. A piece of rubber tubing, fitted with a screw clip, was fixed on the end of the tube containing the cotton wool plug, and, by sucking out a little of the air and then turning the clip, a slight decrease of the pressure of the air inside the flask was obtained. The rubber connection on the other tube was then closed by a clip, and the end of the capillary tube was broken off and carefully sterilised. It was then introduced beneath the surface of the histidine solution which, on opening

the screw clip, passed over into the larger flask. The flask was then sown with an emulsion in sterile Ringer's solution of the organism under investigation, obtained by emulsifying the growth from 20 agar slopes, after 24 hours' incubation. The bacterial emulsion was introduced into the large flask in the same way as the solution of histidine hydrochloride. Samples for analysis were taken from the large flask by creating a slight increase in the pressure of the air in the flask, by blowing gently through the tube containing the cotton wool plug, and then clipping the rubber tube. After closing the rubber connection on the other tube with a clip, the contents of the flask were shaken, and the end of the capillary tube, which was always fused in the Bunsen flame after taking a sample, was broken and sterilised. On opening the clip, the culture media coming over was collected, after rejecting the first 20 or 30 c.c. Care was taken both in sowing the culture media with the organism studied and when samples for analysis were subsequently taken out that no air was allowed to enter the flask except through the cotton wool plug. Bacteriological control showed that, in all the experiments here recorded, there was no contamination. The flasks were incubated at 37°, and, since the tube containing the cotton wool plug was open to the air during incubation, the experiments took place under aerobic conditions.

The following estimations were made on each sample:

I. *Total nitrogen.*

The nitrogen present in 100 c.c. medium was estimated by the Kjeldahl method in the usual way. Since experience showed that there was practically no variation in the total nitrogen during the experiment, determinations were only made at the beginning and end of the experiment. In this, and in the subsequent estimation of free ammonia, the excess of standard acid was always estimated by adding potassium iodide and potassium iodate, and titrating the iodine liberated with standard sodium thiosulphate.

II. *Ammonia and amino acid nitrogen.*

200 c.c. of culture medium was introduced, without previous sterilisation, into a litre Claisen distilling flask, 100 c.c. alcohol, and 10 c.c. of a 10 % suspension of pure lime were added. The free ammonia was distilled off, *in vacuo*, at 40°–45°, and absorbed in an excess of standard $N/10$ acid. The distillation apparatus used was that recommended by van Slyke [1911, 2] for the estimation of the ammonia formed during the hydrolysis of proteins. By this means, one avoids the decomposition into ammonia of readily hydrolysable nitrogen compounds which might be formed by the action of bacteria on histidine (*e.g.* urea). By titration of the excess of acid present, the amount of ammonia formed by the organism studied can be calculated.

After half-an-hour's distillation, the apparatus was disconnected. An excess of glacial acetic acid was added to the Claisen flask, and the solution was concentrated *in vacuo* to a volume of a few c.c. This was then washed out

quantitatively into a 25 c.c. flask, and 2 c.c. of glacial acetic acid were added to prevent any possible action by bacteria which might by this time still be alive, and the whole was made up to 25 c.c. The amino acid nitrogen present was then estimated by van Slyke's [1912] method, 8 c.c. being used for each of the estimations, which were done in triplicate.

All the samples were taken at the same time each day, so that periods represented whole days, and all the estimations on one sample were done on the same day. In the subsequent table, the different forms of nitrogen are expressed as milligrams of nitrogen per 100 c.c. of the medium, and are corrected for impurities due to reagents, etc.

In all the curves given to express graphically the results obtained (pp. 452—455), the following plan has been used:

Curve *B* = ———— represents the amino acid nitrogen (*B*) at different times.

Curve *C* = ... represents the ammonia nitrogen (*C*).

Curve *D* = – – – – – – – – represents the *total* non-amino nitrogen (*D*).

Curve *E* = —— – – —— represents the non-amino nitrogen (*E*).

A consideration of the results shows that, with the exception of *B. proteus vulgaris*, the general *type* of curve is very similar in each case. They all show a gradual decrease in amino acid nitrogen (*B*) and total non-amino nitrogen (*D*), along with a gradual increase in the ammonia nitrogen (*C*) and non-amino nitrogen (*E*). Notably with *B. pyocyaneus*, and to a less extent with *B. proteus vulgaris*, the end point of the reaction is reached much more quickly than with the other organisms. A comparison of the relative *rates* of production of ammonia nitrogen (*C*) and non-amino nitrogen (*E*), and of the decomposition of amino acid nitrogen (*B*) and total non-amino nitrogen (*D*) shows the following interesting differences:

(*a*) With *B. paratyphosus A*, and the two experiments with *B. paratyphosus B*, the *rates* of each of these reactions increases to a maximum which occurs between the twentieth and thirtieth days, and then decreases.

(*b*) With *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*, the *rates* of each of these reactions are all decreasing over the whole course of incubation.

B. proteus vulgaris is sharply differentiated from all the other organisms studied, by the fact that no ammonia is produced from the nitrogen contained in the iminazole nucleus.

A clearer conception of the meaning of the results obtained is gained by comparing the ratios of values obtained by experiment with those which would arise in any hypothetical case. Thus, if the following values are assigned to the letters given:

B = Total decrease in amino acid nitrogen during the course of the experiment, *i.e.* the difference between the initial and final amino acid nitrogen values;

Table I.

Distribution of nitrogen in milligrams per 100 c.c. of medium.

Organism studied	Period of incubation in days	Total nitrogen *A*	Amino acid nitrogen *B*	Ammonia nitrogen *C*	Total non-amino nitrogen *D*	Non-amino nitrogen *E*
B. paratyphosus A	—	51·41	16·70	0·59	34·12	0·72
Expt I (Fig. 1)	9	51·41	14·65	3·53	33·23	3·93
	17	51·41	10·82	8·75	31·84	10·82
	24	51·41	7·72	13·51	30·18	15·44
	31	51·41	5·17	18·42	27·82	17·48
	38	51·41	2·58	22·83	26·00	20·84
	45	51·20	1·02	25·49	24·69	22·65
B. paratyphosus B	—	50·07	16·37	0·51	33·19	0·45
(Birmingham)	8	50·07	14·47	3·39	32·21	3·27
Expt II (Fig. 2)	16	50·07	12·02	7·70	30·35	6·31
	23	50·07	8·79	13·29	27·99	10·41
	30	50·07	4·62	20·69	24·76	15·52
	37	50·07	1·35	26·55	22·17	19·47
	43	49·75	1·22	27·55	20·98	18·54
B. paratyphosus B	—	51·75	16·63	0·67	34·45	1·19
(Schottmüller)	5	51·75	15·36	3·11	33·28	2·56
Expt III (Fig. 3)	19	51·75	7·33	18·13	26·29	11·63
	26	51·75	2·83	24·46	24·46	18·80
	38	51·75	2·39	26·76	22·60	17·82
	73	51·06	1·40	27·67	21·99	19·19
B. faecalis alcaligenes (i)	—	52·21	17·14	0·66	34·41	0·13
Expt IV (Fig. 4)	5	52·21	12·29	9·02	30·90	6·32
	8	52·21	10·41	12·12	29·68	8·86
	14	52·21	7·44	18·68	26·09	11·21
	22	52·21	5·04	23·68	23·49	13·41
	30	52·21	4·24	26·41	21·56	13·08
	57	52·05	4·25	27·75	20·05	11·55
B. faecalis alcaligenes (ii)	—	46·44	15·13	0·21	31·10	0·84
Expt V (Fig. 5)	4	46·44	13·45	3·26	29·73	2·83
	9	46·44	12·09	6·03	28·32	4·14
	16	46·44	9·62	10·58	26·24	7·00
	25	46·44	6·69	17·35	22·40	9·02
	38	46·44	4·24	22·57	19·63	11·15
	72	47·76	1·07	31·38	15·31	13·17
B. pyocyaneus	—	51·60	16·68	0·55	34·37	1·01
Expt VI (Fig. 6)	3	51·60	13·62	9·02	28·96	1·72
	7	51·60	8·52	17·49	25·59	8·55
	12	51·60	4·14	27·27	20·19	11·91
	19	51·60	1·50	35·65	14·45	11·45
	25	51·60	1·03	37·04	13·53	11·47
	33	51·51	0·75	37·61	13·15	11·65
B. proteus vulgaris	—	51·63	16·62	0·66	34·35	1·11
Expt VII (Fig. 7)	5	51·63	10·77	6·20	34·66	13·12
	8	51·63	8·67	8·38	34·58	17·24
	15	51·63	7·42	9·44	34·77	19·93
	23	51·63	6·73	9·93	34·97	21·51
	35	51·60	5·87	10·59	35·14	23·40

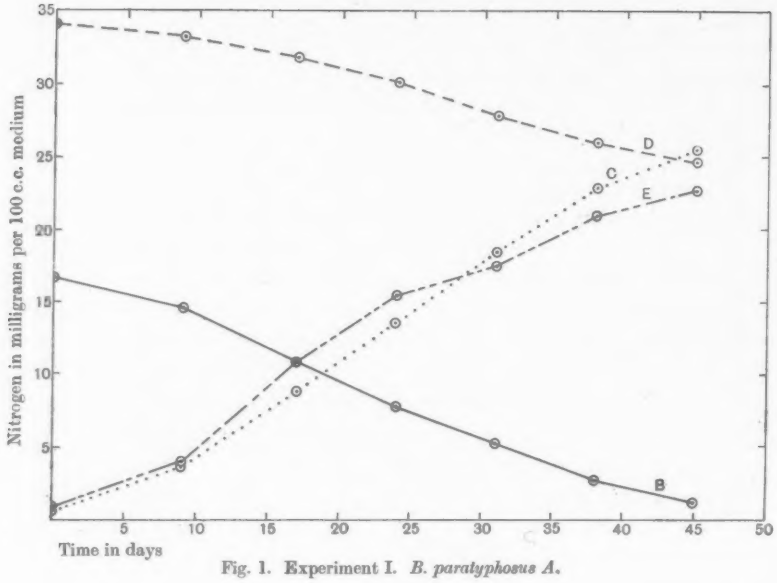


Fig. 1. Experiment I. *B. paratyphosus A.*

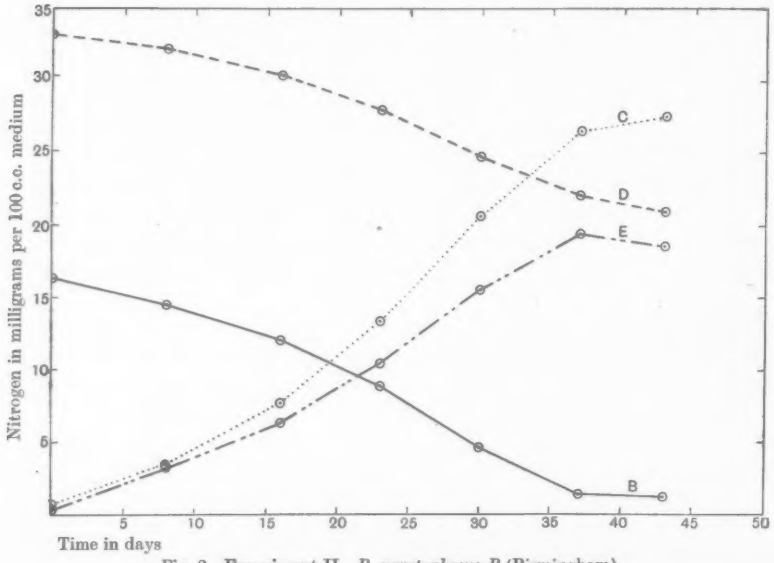


Fig. 2. Experiment II. *B. paratyphosus B* (Birmingham)

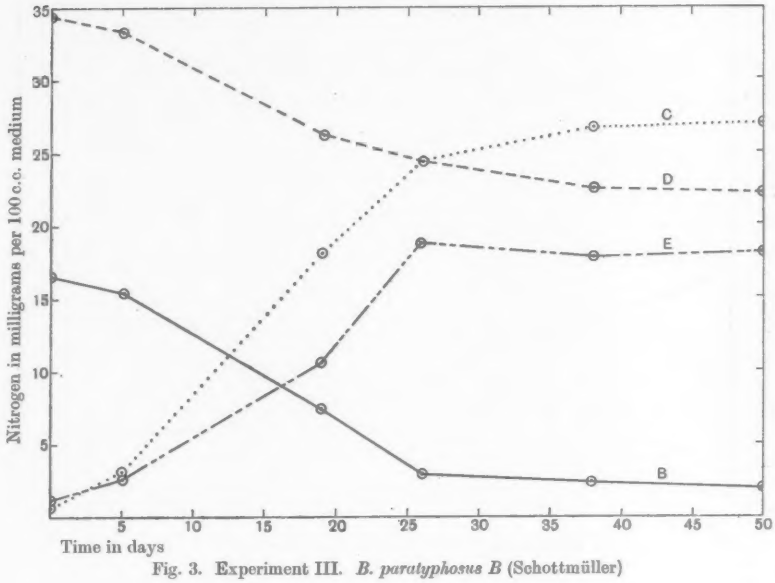


Fig. 3. Experiment III. *B. paratyphosus B* (Schottmüller)

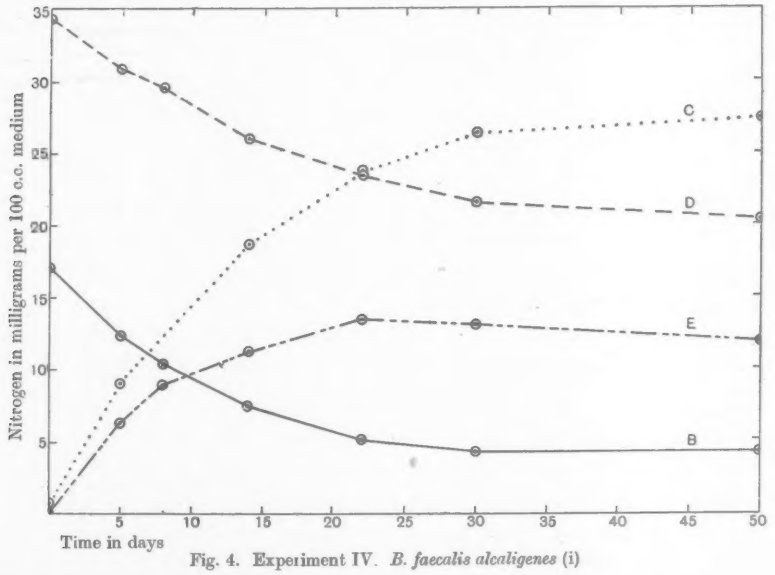


Fig. 4. Experiment IV. *B. faecalis alcaligenes* (i)

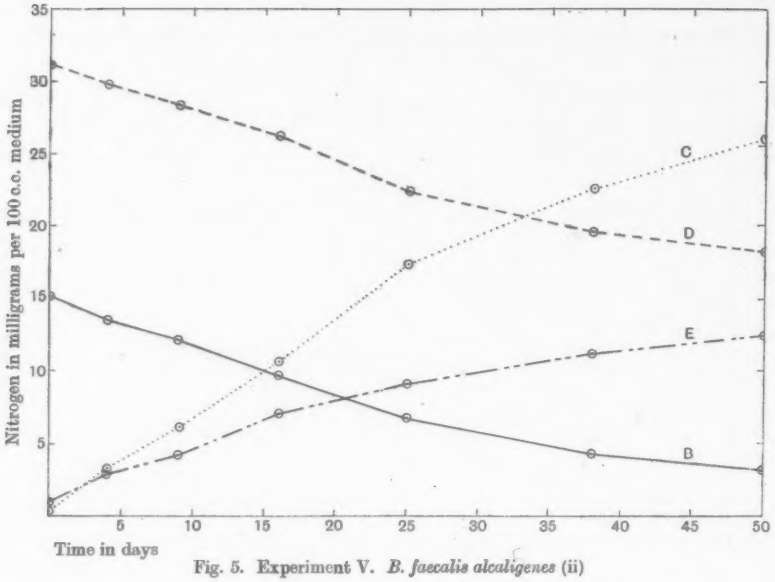


Fig. 5. Experiment V. *B. faecalis alcaligenes* (ii)

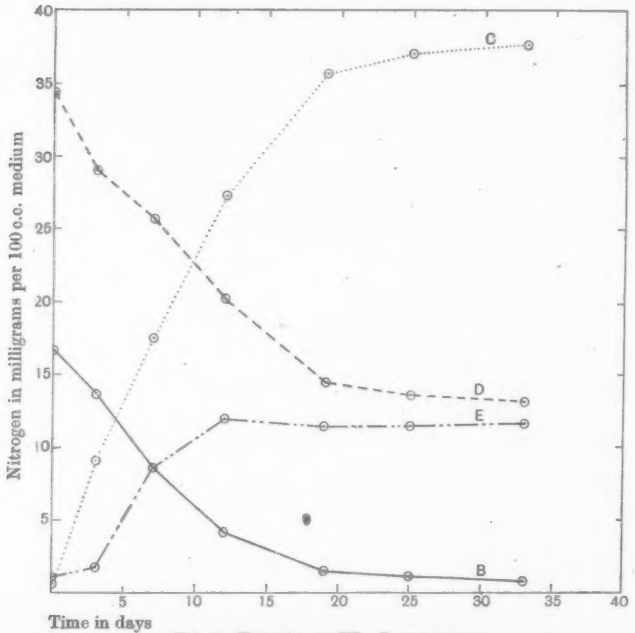


Fig. 6. Experiment VI. *B. pyocyaneus*

C = Total increase in ammonia nitrogen under the same conditions;
D = Total decrease (or increase) in total non-amino nitrogen (as previously defined);
E = Total increase in non-amino nitrogen (as previously defined);
the ratios which these values would have in the following hypothetical cases may be considered:

(i) *Simple de-aminisation of the side chain.* If the amino acid nitrogen in the side chain were converted into ammonia, then

$$\frac{C}{B}=\frac{1}{1},\ \frac{D}{B}=\frac{0}{1},\ \frac{E}{B}=\frac{2}{1}.$$

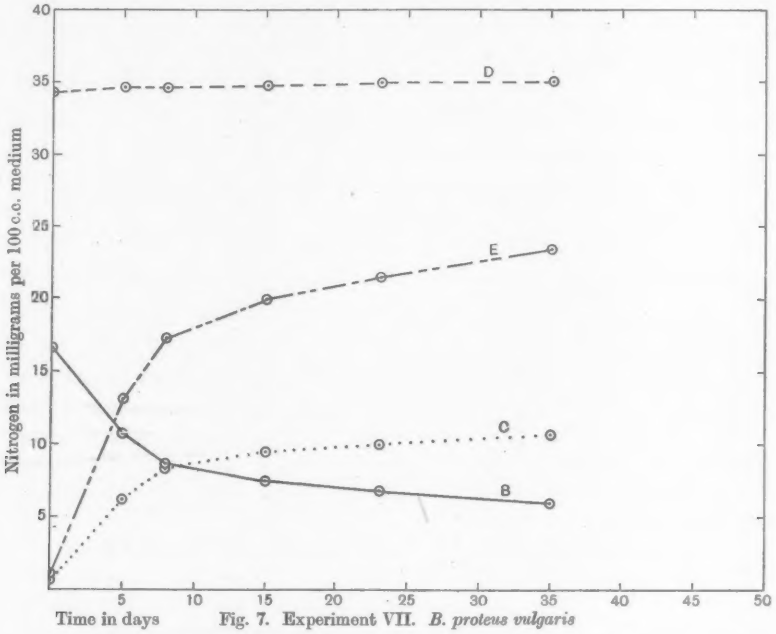


Fig. 7. Experiment VII. *B. proteus vulgaris*

(ii) *Simple decarboxylation of the side chain.* If this reaction alone took place, no ammonia would be formed. The amino acid nitrogen would decrease, since the amine grouping —CH_2—NH_2 does not give any appreciable amount of nitrogen under the conditions employed for estimating amino acid nitrogen.

Then $$\frac{C}{B}=\frac{0}{1},\ \frac{D}{B}=\frac{1}{1},\ \frac{E}{B}=\frac{3}{1}.$$

(iii) *Production of ammonia from both side chain and nucleus.* Since there are two nitrogen atoms in the iminazole nucleus, we may assume that either

(*a*) both nuclear nitrogen atoms and side chain nitrogen would be attacked at the same rate, then

$$\frac{C}{B}=\frac{3}{1},\ \frac{D}{B}=\frac{2}{1},\ \frac{E}{B}=\frac{0}{1};$$

or (*b*) only one of the nuclear nitrogen atoms, in addition to the side chain nitrogen, would be attacked, then

$$\frac{C}{B}=\frac{2}{1},\quad \frac{D}{B}=\frac{1}{1},\quad \frac{E}{B}=\frac{1}{1}.$$

The ratios obtained for the different bacteria investigated, calculated from the results previously given, are summarised in the following table.

[Thus *e.g.* for *B. paratyphosus A*, $\frac{C}{B}=\frac{25{\cdot}49-0{\cdot}59}{16{\cdot}70-1{\cdot}02}=\frac{24{\cdot}90}{15{\cdot}68}=\frac{1{\cdot}59}{1}$.]

Table II.

Organism	$\frac{C}{B}$	$\frac{D}{B}$	$\frac{E}{B}$
B. paratyphosus A	$\frac{1{\cdot}59}{1}$	$\frac{0{\cdot}60}{1}$	$\frac{1{\cdot}40}{1}$
B. paratyphosus B (Birmingham)	$\frac{1{\cdot}78}{1}$	$\frac{0{\cdot}81}{1}$	$\frac{1{\cdot}19}{1}$
B. paratyphosus B (Schottmüller)	$\frac{1{\cdot}77}{1}$	$\frac{0{\cdot}82}{1}$	$\frac{1{\cdot}18}{1}$
B. faecalis alcaligenes (i)	$\frac{2{\cdot}10}{1}$	$\frac{1{\cdot}11}{1}$	$\frac{0{\cdot}886}{1}$
B. faecalis alcaligenes (ii)	$\frac{2{\cdot}22}{1}$	$\frac{1{\cdot}12}{1}$	$\frac{0{\cdot}877}{1}$
B. pyocyaneus	$\frac{2{\cdot}33}{1}$	$\frac{1{\cdot}33}{1}$	$\frac{0{\cdot}67}{1}$
B. proteus vulgaris	$\frac{0{\cdot}985}{1}$	$\frac{0{\cdot}073}{1}$	$\frac{2{\cdot}07}{1}$

From a consideration of the results given in Table II, and from the different graphs, it seems justifiable to draw the following conclusions:

(i) *B. proteus vulgaris* alone, of the organisms investigated, cannot produce ammonia from either of the nitrogen atoms in the iminazole nucleus, although it does so from the nitrogen atom in the side chain. This is probably due to one of two causes:

(*a*) The inability of the organism to open the ring. If this explanation, which seems the more probable, is correct, the reason would appear to be that the enzyme, which enables the other bacteria investigated to open the iminazole ring, is not present in *B. proteus vulgaris*. The evidence offered in this paper seems to point, in fact, to the presence in many bacteria of a specific enzyme (perhaps more than one), which has the power of splitting open the iminazole ring, and producing ammonia from the nitrogen atoms contained in the opened ring. The ease with which this change is brought about by bacteria is in marked contrast to the well-known stability of the iminazole ring towards most chemical reagents.

(*b*) The possibility, an improbable one, that although *B. proteus vulgaris* can open the iminazole ring it cannot convert the nitrogen contained in the ruptured ring into ammonia.

(ii) None of the bacteria produced simple decarboxylation of the histidine, and it is to be regretted that for the sake of completeness it was impossible

under conditions prevailing at the time, to obtain a culture of one of the well-known decarboxylating bacteria.

(iii) *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. paratyphosus A*, and *B. paratyphosus B* produce ammonia both from the nitrogen in the side chain and in the nucleus. All these bacteria attack the histidine molecule as a whole by a series of reactions which proceed concurrently, and produce a complete breakdown of at least a portion of the iminazole ring. Two of them, *B. faecalis alcaligenes* and *B. pyocyaneus*, produce ammonia from the ring more quickly than they produce it from the side chain. Since the ratio $\frac{D}{B}$ is greater than $\frac{1}{1}$ and since the ratio $\frac{C}{B}$ is greater than $\frac{2}{1}$ with these two organisms, it follows that ammonia is formed by each of them from each of the three nitrogen atoms in the histidine molecule. The fact that the ratio $\frac{E}{B}$ is in the one case $\frac{0{\cdot}88}{1}$ and in the other $\frac{0{\cdot}67}{1}$ probably explains the previously described failure to isolate any iminazole compound from the action of *B. pyocyaneus* on histidine.

A comparison of the ratios obtained with *B. paratyphosus A* and *B. paratyphosus B* shows that these two organisms, and more particularly *B. paratyphosus A*, produce ammonia from the side chain more quickly than they do from the ring since the ratio $\frac{D}{B}$ is in each case less than $\frac{1}{1}$. The fact that the ratio $\frac{E}{B}$ for *B. paratyphosus A* is relatively large $\left(\frac{1{\cdot}40}{1}\right)$ explains why such a good yield of urocanic acid was obtained as a result of the action of this organism on histidine, since $\frac{E}{B}$ represents the ratio of end products, other than ammonia, to histidine decomposed.

It is of interest to note that though the value of any one of the ratios $\frac{C}{B}, \frac{D}{B}, \frac{E}{B}$ is different for each of the different bacteria investigated, they show very good agreement for different strains of the same organism (*e.g.* for two different strains of *B. paratyphosus B* and of *B. faecalis alcaligenes*).

Summary.

The aerobic breakdown of histidine by bacteria (*B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*) was studied quantitatively.

The results obtained showed that:

(i) *B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, and *B. pyocyaneus* can attack, and produce ammonia from, the nitrogen atoms in both the side chain and the iminazole nucleus of the histidine molecule, thus proving that they are able to open the iminazole ring.

(ii) With *B. proteus vulgaris*, ammonia is formed only from the side chain nitrogen, and it is very probable that this organism is unable to open the iminazole ring.

I wish to take this opportunity of expressing my gratitude and indebtedness to Professor F. Gowland Hopkins for much valuable criticism and advice during the progress of this research.

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XL. NITROGEN METABOLISM IN *SACCHAROMYCES CEREVISIAE.*

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Introduction.

During the last few years, the assimilation of nitrogen by the lower organisms of the vegetable kingdom has formed the subject of an ever-increasing number of investigations, the results of which have rendered evident the great importance of this question in its bearing on the problems of both pure and applied chemistry.

Of these researches, not the least suggestive, from a purely chemical, as well as from an industrial, standpoint are those of Felix Ehrlich and his collaborators, who succeeded in establishing the fact that the "fusel oil" of the distiller is produced by the action of yeast on the amino-acids in the wort, yielding the corresponding alcohols, such amino-acids being the degradation products of the proteins originally present.

It is not necessary to trace the history of this theory, first put forward by A. Müller in 1857, and either disregarded or discredited until Ehrlich in 1903 proved that the α-amino-acids when treated with yeast in a saccharine solution give the corresponding alcohols.

The object of the work described in the present communication was the closer investigation of those factors which affect the nitrogen metabolism in yeast, not only from the point of view of the assimilation of the nitrogen detached from the amino-acid during its conversion into an alcohol, but also from the point of view of an apparent excretion of nitrogen which takes place with yeast under certain circumstances.

Part I. Investigation of the factors which influence the assimilation of nitrogen from amino-acids by yeast.

Theoretical.

In view of the important and fundamental character of the work of Ehrlich, it was deemed advisable to repeat the final experiments of this

worker, and the results obtained with leucine confirmed Ehrlich's conclusions, namely, (1) that the amount of nitrogen lost by the amino-acid equals the amount gained by the yeast; (2) that the amount of nitrogen lost by the liquid corresponds to the amount of amyl alcohol formed; (3) that the amount of alcohol formed corresponds to about half the weight of leucine introduced initially into the solution. An extension of the same method to the desamination of asparagine gave further confirmatory results, but here, as would be expected, analysis of the resultant liquid failed to reveal the presence of any of the higher alcohols, a result quite in agreement with the theory of Ehrlich, according to whose equations the only product formed by the extraction of nitrogen from asparagine would be ethyl alcohol.

The main conditions under which these experiments were carried out may be briefly summarised as follows:

(1) Yeast of low nitrogen content.

(2) Non-multiplying yeast.

(3) Large amount of sugar available for fermentation.

The first of these conditions is contingent on the statement of Ehrlich that a yeast of low nitrogen content has a greater assimilatory power for nitrogen than has one in which the percentage of protein matter is high, and a comparison of two yeasts of the same race but containing different percentages of nitrogen showed that there was a small but distinct difference between the amounts of nitrogen assimilated by the yeasts, the comparison being made with yeasts in which the difference in the nitrogen content was artificially induced by previous treatment.

The amount of yeast added to the saccharine amino-acid solution in Ehrlich's experiments precluded the possibility of cell reproduction and it consequently appeared probable that the conclusions drawn from these experiments might not be valid if the yeast were in a state of active cell multiplication during the time the nitrogen assimilation was taking place.

To elucidate this point saccharine amino-acid solutions containing the inorganic salts commonly employed as yeast nutriment (Pasteur) with the exception of the ammonium salts—the amino-acid taking their place—were seeded with a pure culture of *S. cerevisiae* in such amounts that the yeast concentration in three experiments was 1, 10, and 100 cells per unit volume respectively, and the results so obtained are of a most interesting character.

In the first place they show that an excess of yeast extracts the maximum total amount of nitrogen, that is to say, that, from equal volumes of an amino-acid solution a suspension of 100 cells/T[1] assimilates a much greater amount of nitrogen than a suspension of 10 cells/T. On the other hand, regarded from the point of view of a single cell, the greater the cell concentration the less the amount of nitrogen assimilated by each cell (calculated on the number of cells found in the solution at the end of the experiment).

[1] T is the symbol for the standard unit volume of 1/4000 cubic millimetre.

The following figures (mean of three typical experiments) demonstrate this:

Table I.

Rate of seeding	G. nitrogen extracted per unit cell	
	Leucine	Asparagine
100 cells/T	$3{\cdot}37 \times 10^{-4}$	$11{\cdot}53 \times 10^{-4}$
10 cells/T	$8{\cdot}87 \times 10^{-4}$	$12{\cdot}85 \times 10^{-4}$
1 cell/T	$31{\cdot}95 \times 10^{-4}$	$31{\cdot}45 \times 10^{-4}$

The figures (see experimental section) show a noticeable lack of increase in the number of cells in those experiments seeded at the rate of 1 cell/T.

There are apparently three factors which might account for this, (1) lack of oxygen, (2) effect of the products of fermentation, (3) effect of some substance originally present.

Experiments proved the first factor to be of no practical importance, for aerated and non-aerated fermentations yielded similar results.

The second factor is more difficult of consideration. On the assumption—a very simple one—that the decrease in the amount of nitrogen is an indication of the amount of action which has taken place, and that consequently it is proportional to the quantity of any inhibitant formed, it follows that (taking concrete examples from the results recorded in the experimental part) in two leucine experiments (Tables III and IV) seeded with 10 (B) and 1 (C) cell per unit volume respectively, the inhibitant should be in the ratio 0·0317 to 0·0195, a concentration reached when the number of cells in these two experiments were 32 and 7 respectively. Thus it follows that each cell in B would be influenced by 0·0317/32 parts of inhibitant whilst each cell in C had to sustain the action of 0·0195/7 parts, that is 9·9 parts in B to 28·0 parts in C. Therefore if such a body is formed there is here a reason for the lack of increase in C, the experiment seeded with 1 cell per unit volume. It should be noted, however, that careful analysis of the fermented liquids failed to show the presence of any inhibitory body.

With regard to the third factor it must be remembered that amino-acids although providing food for yeast, exert retarding influences on the reproductive capacity of the yeast cell, and it may also be that these amino-acid preparations may contain minute traces of bodies which in the light of modern knowledge may have an inhibitory effect on the reproductive activity of the yeast. It has been shown that in the case of antiseptics, a certain concentration will stop the growth of an approximately definite number of organisms, but if the number of organisms be increased there will be a limiting value for the amount of antiseptic per cell, below which value reproduction can take place. It may be that the preparations of amino-acid used act in a similar manner, and that 100 cells/T gives in the above experiments a concentration of amino-acid below the limiting value, while the 1 cell/T is above this value and consequently reproduction is retarded.

Yet one other point of interest is attached to this series of experiments, namely, the irregularity of the final nitrogen coefficient of the yeast; but a comparison shows that the final value of this coefficient is apparently connected in some way with the reproductive activity of the yeast, and that for one set of factors the yeast has apparently a specific nitrogen coefficient. (Table VIII, p. 468.)

Experimental.

1. *Fermentation of leucine and asparagine solutions.* The preliminary experiments which were carried out during the course of these investigations were, as has already been stated, conducted along the lines given by Ehrlich himself, and consisted essentially of the fermentation of sugar solutions containing amino-acids, by yeasts of low nitrogen content. The results demonstrated clearly the truth of the conclusions which this worker himself drew. Applying the same method of experiment to the fermentation of asparagine, further confirmatory results were obtained, but in this case, as would be expected, careful analysis of the resultant liquid failed to show the presence of any higher alcohol.

2. *Effects of original nitrogen coefficient of yeast.* In order to test the statement that a yeast of low protein content extracts more nitrogen than one in which the amount of protein is high, a comparison was made between yeasts of different protein content. The seed-yeasts were prepared, in the one case by allowing the yeast to ferment without cell-multiplication in a wort rich in nitrogen, and in the other case by growing a sample of the same yeast in the wort diluted with water, the yeast being seeded at the rate of 10 cells per unit volume T. The yeasts thus prepared were finally washed and introduced at the rate of 10 cell/T into a 10 % cane sugar solution containing 0·1 % nitrogen as asparagine, and the liquid maintained at 37°. At the end of two days the fermentation was finished, and the following results were obtained.

Table II.

Seed yeast		Total nitrogen in solution		
Condition	% nitrogen	Before	After	% nitrogen assimilated
Low	6·85	0·1000	0·0748	25·20
High	9·32	0·1000	0·0780	22·00

The distinct, although small, difference between the two numbers in the final column confirms Ehrlich's statement that the amount of nitrogen extracted is to some extent related inversely to the amount of protein originally present in the organism. At least this is so for yeasts of an *artificially* high or low nitrogen content.

3. *Effects of cell reproduction on nitrogen extraction.* The effect of active cell multiplication on nitrogen absorption was investigated by seeding a nutrient amino-acid solution with a pure culture of *S. cerevisiae*, the yeast being added to the solution as a suspension in sterile water, in such volume

that the resultant mixture contained in three series, 1, 10, and 100 cells/T. The composition of the nutriment itself was:

Cane sugar	10·00 %
Potassium di-hydrogen phosphate	0·20
Calcium phosphate	0·02
Magnesium sulphate	0·02
Nitrogen (as leucine or asparagine)	0·10
Water to	100·00

Tables III and IV give the results obtained in two such series.

Table III.

			Before fermentation			After fermentation			Difference	
Source of N	Rate of seeding per unit volume		*a* Total N	*b* N in yeast	*c* N in acid	*d* Total N	*e* N in yeast	*f* N in acid	*g* Increase N in yeast	*h* Decrease N in acid
Leucine	100		0·3256	0·2122	0·1134	0·3227	0·2444	0·0783	0·0322	0·0351
Aspar.			0·3991	0·1764	0·2227	0·3976	0·2830	0·1146	0·1066	0·1081
Leucine	10		0·1346	0·0212	0·1134	0·1280	0·0463	0·0817	0·0251	0·0317
Aspar.			0·2403	0·0176	0·2227	0·2378	0·0561	0·1817	0·0385	0·0410
Leucine	1		0·1155	0·0021	0·1134	0·1206	0·0267	0·0939	0·0246	0·0195
Aspar.			0·2245	0·0018	0·2227	0·2254	0·0280	0·1974	0·0262	0·0253

Table IV.

		No. of cells per T				N coefficient of yeast × 10^4			N increase per unit cell × 10^4
Source of N	Rate of seeding per unit volume	*k* Before	*l* After	*l*/*k* Coefficient of multiplication	*m* No. of new cells	*n* Before	*p* After	*q* Difference	*t*
Leucine	100	97·8	94·3	—	—	21·7	25·9	+ 4·2	3·41
Aspar.		97·8	93·4	—	—	18·0	30·3	+12·3	11·50
Leucine	10	9·8	32·0	3·28	22·2	21·7	14·5	− 7·2	7·84
Aspar.		9·8	33·1	3·37	23·3	18·0	17·0	− 1·0	11·60
Leucine	1	1·0	6·9	6·90	5·9	21·7	38·7	+17·0	35·65
Aspar.		1·0	7·9	7·90	6·9	18·0	35·5	+17·5	33·16

In the above results the small increase in the number of cells in those experiments seeded at the rate of 1 cell/T is very noticeable, and the following experiments were conducted in order to get some idea of the state of the cells at the termination of the fermentation.

4. *Final condition of the yeast cell in experiments seeded at* 1 *cell*/T. A nutrient asparagine solution (of composition given above) was seeded at the rate of 1 cell/T, and allowed to ferment at 37° in the usual manner. At the conclusion of the fermentation, a count of the number of yeast cells was made, the yeast separated by filtration and the filtrate distilled *in vacuo* at 40°. The residue was then dissolved in 10 % sucrose solution, and the solution made up to the original volume of the asparagine solution, and finally treated with the previously separated yeast. On the completion of the second fermentation, a count of the cells was again made, with the following results:

Table V.

Fermentation	No. of cells per unit volume		Coefficient of multiplication
First	1·10	9·20	8·36
Second	9·00	31·40	3·49

This then conclusively proves that the yeast in the initial experiment (seeded at the rate of 1 cell/T) was not killed, but that its *reproductive activity had been retarded*.

5. *Effect of aeration on nitrogen extraction by yeast.* Fermentations were conducted as previously described (leucine and asparagine being added as the sources of nitrogen in two parallel series), except that the fermenting liquid was aerated by the passage of a stream of sterile air for a short time each day. The results are given in the following table, together with those previously shown in Table III for purposes of comparison. Experiments with asparagine gave confirmatory results.

Table VI.

Conditions		Nitrogen in solution g. per 100 cc.			No. of cells per unit volume			Coefficient of multiplication
Rate of seeding	Aeration	Before	After	Decrease	Before	After	Increase	
100	Non-Aerated	0·1134	0·0783	0·0351	97·80	94·30	—	—
	Aerated	0·1094	0·0698	0·0396	99·00	98·00	—	—
10	Non-Aerated	0·1134	0·0817	0·0317	9·80	32·00	22·20	3·28
	Aerated	0·1094	0·0786	0·0308	9·90	33·20	23·30	3·35
1	Non-Aerated	0·1134	0·0939	0·0195	1·00	6·90	5·90	6·90
	Aerated	0·1094	0·0914	0·0180	1·00	8·40	7·40	8·40

6. *Effect of the conditions of cell reproduction on the final nitrogen coefficient of the yeast.* The following table shows that there is apparently some connection between the final nitrogen coefficient of the yeast and the conditions of cell reproduction, for the mean of the three quoted experiments seeded at 100 cells/T is 0·0026, at 10 cells/T is 0·0016, and at 1 cell/T is 0·0036.

Table VII.

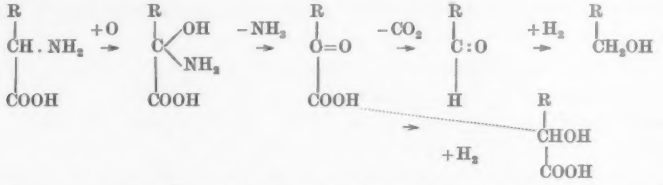
	Nitrogen coefficient of yeast × 10^4			
	Initial	Final		
	Experiments	*A*	*B*	*C*
Amino-acid	*A*, *B* and *C*	100 cells/T	10 cells/T	1 cell/T
Leucine	21·7	25·0	14·0	38·0
,,	18·0	24·0	16·0	35·0
Asparagine	18·0	30·0	17·0	35·0

Part II. Consideration of the theory of the mechanism of the removal of nitrogen from amino-acids by yeast.

Fifteen years ago Ehrlich [1905] put forward his theory of the degradation of amino-acids by yeast, explaining the action as follows. The (NH_2) groups of the amino-acids are first split off from the parent molecule by the action of some desaminating enzyme, with the formation of the corresponding hydroxy-acid. The ammonia thus liberated is assimilated by the yeast and probably used in the construction of protein matter. At the same time the hydroxy-acid is subjected to what is presumably the ordinary zymatic action of the yeast, namely, the liberation of carbon dioxide and consequent formation of an alcohol, an action analogous to the supposed decomposition of lactic acid

under similar circumstances to carbon dioxide and ethyl alcohol. This theory states then that a hydroxy-acid is the intermediate product of the desamination.

Six years later, however, some work was published by Neubauer and Fromherz [1911] which carries Ehrlich's work a step further, in that they isolated a ketonic acid as the intermediate product of the action. They modify the theory of the action, for, in their opinion, the degradation is represented graphically as follows:



According to both these theories, the degradation of the amino-acid molecule is primarily caused by a hydrolysing enzyme, followed by a zymatic one, but Neubauer and Fromherz's explanation also requires the presence in the yeast of two others, an oxidising and a reducing enzyme.

The intermediate body, according to Ehrlich, is a hydroxy-acid, and Neubauer and Fromherz have succeeded in separating such a body, although they state it to be a reduction bye-product of the true intermediate body, the ketonic acid.

In any case the alcohol corresponding to the amino-acid, as shown in the above formulae, results: tyrosine, phenyl-amino-acetic acid, serine, and α-amino-α-methylbutyric acid, all have been shown by Ehrlich to yield the expected alcohol.

Hans Pringsheim [1906, 2] has also studied the desamination of the amino-acids more particularly in connection with the Mucors, and concludes from his results that for nitrogen to be assimilated from a complex molecule the group (—CO—CH—NH) must be present, and as proof of this gives the following list of bodies which he has succeeded in decomposing by moulds: glycine, alanine, leucine, tyrosine, aspartic acid, phenylglycine, allantoin, phenylalanine, and hippuric acid. He further states that a long side chain favours such a degradation.

This production of alcohols from amino-acids is, however, quite distinct from the ordinary zymatic action of the yeast, for both Ehrlich [1906] and Pringsheim [1906, 1] have proved that no amyl alcohol is formed from leucine by the action of active "acetone yeast" in the presence of sugar.

Thus the following conclusion naturally presents itself: There are two—in some ways different—actions taking place, (1) the desaminating action proper, and (2) the zymatic action. It appears probable that could these two actions be separated some light would be thrown on the extraction of nitrogen from amino-acids by yeast, and therefore it may be that by suppressing those

actions connected with the zymatic activity the presence in the yeast cell of a desaminating enzyme could be proved.

Effront [1908] apparently succeeded in doing this to a certain extent, for he found that in the decomposition of amino-acids by yeast in an alkaline medium he could demonstrate the presence of an enzyme, amidase, which under certain conditions liberates the whole of the nitrogen of amino-acids as ammonia, leaving various acids as the result of the action. For example, the whole of the amino-acid nitrogen of asparagine and part of the nitrogen of the yeast material itself are set free as ammonia in 96 hours. He also states that the enzyme can be obtained in solution.

Experiments carried out along the lines described by Effront showed two fertile sources of error, namely, bacterial growth and the presence in the liquid to be distilled of free alkali, both of which would nullify any results obtained [Effront, 1909].

The conditions of experiment are such that the yeast is dead, and a further proof that the action is quite independent of the life of the yeast was obtained by the use of dried, finely ground yeast instead of the pressed yeast before employed, the results showing that a little less than 75 % of the nitrogen was liberated as ammonia (Table IX, p. 469).

Thus the presence in the yeast cell of a desaminating enzyme, independent of the life of the yeast cell itself, seems to be proved, although it has been found impossible to extract the enzyme from the yeast in the manner described by Effront (*Experimental Section*, § 2).

Besides this desaminating ferment, amidase, however, another enzyme which causes hydrolysis analogous to the supposed hydrolysis by amidase was discovered in yeast by Shiga [1904]. This enzyme was arginase, and had previously been found in animal tissue by Dakin [1904] and its action more fully investigated by Kossel and Dakin [1904, 1, 2]. Its specific activity is the hydrolysis of arginine to ornithine and urea, although experiment proved the action to go further, the urea itself being hydrolysed.

Kossel and Dakin not only discovered this enzyme, but they also succeeded in separating it in a solid form from solution by precipitation [Kossel and Dakin, 1904, 3], and as the tissues which contain arginase act vigorously on asparagine, glutamic acid, etc., it seemed possible that this enzyme was the one at work in the experiments conducted by Effront. This possibility was more or less discountenanced by Effront's observation that amidase would not act in an acid medium, while the arginase of Kossel and Dakin is extracted by and is active in a 0·2 % acetic acid solution. Attempts at acid extraction, however, also proved abortive, and one is forced to the conclusion that the enzyme amidase is either in an insoluble state in its original form in the yeast tissue, or that it depends for its action upon a coferment which cannot be extracted by the means employed above.

If the action were a simple hydrolysis, malic acid should result from asparagine, but Effront states that the chief product of the action is propionic

acid. This being the case, the action must be a complex one and zymatic and reducing enzymes must also take part in the degradation. That yeast can, under suitable conditions, reduce the hydroxy-group of malic acid was proved by A. Fitz[1] who obtained calcium succinate from calcium malate. Reduction is also necessary in the theory of Neubauer and Fromherz.

Analysis of the liquid resulting from the action of yeast on asparagine under Effront's conditions revealed the presence of volatile fatty acids, apparently acetic and propionic, together with a non-volatile acid corresponding in molecular weight with malic which was present in an amount equal to about 60 % of the asparagine destroyed.

Presuming this removal of ammonia to be the first step in the fermentation of amino-acids, then malic acid—or ammonium malate—should be fermentable by yeast to yield ethyl alcohol alone. Experiment proved that under normal conditions of fermentation malic acid was unattacked by yeast, a result confirmed by some work of Oskar Emmerling [1899: reference to which was not found until after the work was completed] who found that while a pure culture of *B. lactis aerogenes* fermented malic acid to form succinic and formic acids with a little carbon dioxide and ethyl alcohol, pure cultures of brewery yeast had no action on malic acid, the apparent action of ordinary cultures being due to bacterial contamination.

It was thought, however, that the ammonium salt might act in an altogether different manner, for salts are often attacked while the acids themselves resist the action, *e.g.*, it has been stated that calcium malate is reduced by yeast to calcium succinate, and it seemed quite possible that the ammonium salt might serve as a source of nitrogen for the yeast cell. This was found to be the case, for, in the presence of sugar, yeast attacked ammonium malate, ethyl alcohol being the only product of the fermentation revealed by analysis of the resultant liquid. Effront states that the chief product of the action of amidase on asparagine is propionic acid, and consequently were the action of amidase the first step in the fermentation of this amino-acid then ammonium propionate should also be fermentable. This proved to be the case, although only to about half the extent of ammonium malate but quite sufficient to preclude the possibility of drawing any definite conclusions as to the rôle of malic acid in the fermentation of amino-acids.

Experimental.

1. *Desamination by yeast in alkaline solutions.* The method of conducting the desamination of amino-acids by amidase as given by Effront is as follows: 2 g. of asparagine and 10 g. of pressed yeast are mixed together with a little water, 6 cc. of *N* NaOH are then added and the mixture made up to 100 cc. with water. The ammonia formed as a result of the action of amidase on the

[1] Richter's *Organic Chemistry* (Smith), **1**, 488. I cannot find reference to this work, the nearest being that of A. Fitz [1879] who found that certain schizomycetes acted upon malic acid to produce succinic and acetic acids.

asparagine is determined at intervals in a portion of the filtered liquid by distilling *in vacuo* with magnesia; controls of asparagine alone and of yeast alone are carried out concurrently.

The preliminary experiments were carried out in this manner, but as stated above it was found that abnormal results were obtained. Consequently in the following experiments toluene was in all cases added as an antiseptic and previous to distillation the sodium hydrate was neutralised by sulphuric acid[1]. The following are typical results:

Table VIII.

Initial nitrogen in liquid	After 120 hours action		
	Total N	Ammoniacal N	% of initial N as NH_3
1·1200	1·1295	0·8064	72
1·4500	1·1600	0·8477	74

2. *Attempts to obtain amidase in alkaline solution.* In a second parallel experiment the liquid was filtered after 72 hours and the filtrate divided into two portions; to the one 5 g. of asparagine were added, whilst the other was boiled to serve as a control. The liquids were kept at 37° for three days when the free ammonia was estimated, with the result that the control liquid was found to contain the same amount as the test itself, a result directly in opposition to the statement of Effront that under such conditions the enzyme is easily extracted.

3. *Attempts to obtain amidase in acid solution.* About 100 g. of pressed yeast were ground in a mill and extracted with 100 cc. of 0·2 % acetic acid for three days at 37°. The liquid was then filtered, and the filtrate, presumably containing the enzyme, divided into two parts and treated as in the alkaline extractions. The results were quite negative.

4. *Proof that the action of amidase is independent of the life of the cell.* An amount of dried yeast, finely ground, corresponding to the amount of pressed yeast used in the previous experiments was introduced into the amino-acid solution, and the action allowed to proceed as in the cases already described. The results obtained are given in Table IX.

[1] The form of distillation apparatus used was one devised by Brown [1903, 1] a modification of the Longi apparatus as used in the Sachsse-Schloesing-Longi method of ammonia estimation.

A great difficulty was at first experienced in these distillations by reason of the vigorous frothing which occurred when the pressure was reduced to about 50 mm. (at 40°) or when the temperature reached 35° if the pressure was previously reduced to 20 mm. It was found, however, that after raising the temperature to 40° and keeping the pressure at 100 mm. for about 15 to 20 minutes, the pressure could be diminished and the liquid would boil quite normally. The distillations were conducted at 40° and 12–15 mm. pressure. As a safeguard against sudden frothing carrying the liquid over into the receiver, a tap was inserted in the apparatus between the hot water jacket and the receiver so that on the slightest indication of frothing, the pressure in both the distillation flask and receiver could be immediately increased. It also served as a means of keeping the vacuum at the desired pressure in the first part of the distillation.

Table IX.

Time in hours	Total N in liquid	Total N as NH_3 in liquid	% nitrogen as NH_3
24	1·1199	0·3927	35·06
48	1·1200	0·7707	68·80
120	1·1205	0·8069	72·00

5. *Estimation of the volatile acids in resultant liquids.* The liquid, after filtration, was acidulated with sulphuric acid and distilled with additions of water as the liquid in the distilling flask became concentrated. The distillate was subsequently treated with an amount of baryta solution sufficient to neutralise the acid present, and the neutral liquid, after the separation of a certain amount of precipitate, evaporated to dryness in a weighed dish. The residue, after weighing, was treated with sulphuric acid and heated in order to remove the volatile acids. From the weight of sulphate obtained and the amount of barium salt decomposed the molecular weight of the volatile acid was calculated.

Table X.

Weight of barium salt taken	Weight of $BaSO_4$	% Ba in salt	Mol. weight of acid if monobasic
1·7316	1·5448	52·50	64·40
1·0844	0·9581	51·90	65·80

The percentage of Ba in barium propionate is 48·5 and in the acetate 53·8; therefore the above results appear to suggest that the volatile portion of the products of the desamination of asparagine is a mixture of acetic and propionic acids.

6. *Estimation of the non-volatile acids in resultant liquid.* With regard to the non-volatile portion of the acid products it was found a matter of considerable difficulty to obtain a reliable method of estimation. Finally the following method of procedure was decided upon. The experimental liquid was neutralised with sulphuric acid heated to boiling point and treated with an excess of precipitated calcium carbonate, and after boiling for about seven minutes filtered. The clear filtrate was concentrated and filtered to remove any calcium succinate which separated out, and then treated with about ten times its volume of 95 % alcohol. This precipitated any calcium malate, which is practically insoluble in 85 % alcohol. It was found that a convenient method of procedure was to filter through a Gooch crucible, for the salt could then be easily taken into solution again if necessary, or transferred to an evaporating basin for decomposition with sulphuric acid.

The results given below tend to show that a considerable amount of malic acid is produced in the desamination of asparagine by yeast in the conditions of experiment described.

Table XI.

Weight of Ca salt taken	Weight of $CaSO_4$	% Ca in salt	Mol. weight of acid
0·6166	0·4896	23·36	132·8
0·7392	0·5897	23·48	133·5

Calculated for calcium malate $C_4H_4O_5Ca$. Ca 23·25 %.

Molecular weight of malic acid = 134.

Total weight of calcium salt obtained from 2 g. asparagine = 1·3558 g.

Weight of asparagine corresponding to Ca salt obtained = 1·18 g.

Amount of asparagine decomposed (by nitrogen) = 1·623 g.

7. *Fermentation of malic acid and ammonium malate.* Investigation of the power of yeast to ferment malic acid was conducted on a solution containing 1 % malic acid and 10 % cane sugar with pure culture yeast, but the results showed that no acid was destroyed. In the case of the ammonium salt, the procedure was as follows: 1·0 g. malic acid was dissolved in water and the solution neutralised by ammonia; 20 g. of sugar and 20 g. of pressed yeast were added and the mixture made up to 250 cc. The solution was kept at 37° and a vigorous fermentation ensued which was practically over in three days. The progress of the action was followed in two ways (1) by estimation of the amount of nitrogen extracted from the liquid; (2) estimation of the decrease in the amount of malic acid present. Both series of determinations were carried out in the manner previously described, and yielded the results shown in Table XII which prove that ammonium malate is fermented by yeast.

Table XII.

	(*a*) Nitrogen determinations			(*b*) Acid determinations		
Time in hours	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ca salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0·1127	0·6762	Nil	0·7066	0·6902	Nil
24	0·0978	0·5868	13·20	0·6160	0·6017	12·82
96	0·0801	0·4806	28·90	0·5081	0·4964	28·08

8. *Products of the action of yeast on ammonium malate.* After the separation of the malic acid precipitate the filtrate was carefully evaporated to dryness at 40° *in vacuo*. This solution should contain ammonium carbonate (by the double decomposition of calcium carbonate and ammonium malate) and the calcium salt of any acid formed during the fermentation. The residue therefore of this solution was gently heated, with a result that practically the whole of it decomposed, proving it to be ammonium carbonate. The very slight amount of residue left undecomposed was quite inestimable.

Thus it appears that the only product of the fermentation of ammonium malate is ethyl alcohol.

9. *Fermentation of ammonium propionate.* The results of experiments are given in Table XIII, which show that this salt is also decomposed by yeast. The products of the action were not analysed.

Table XIII.

	(*a*) Nitrogen determinations			(*b*) Acid determinations		
Time in hours	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ba salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0·0916	0·5954	Nil	0·9350	0·6013	Nil
24	0·0875	0·5685	4·52	0·8868	0·5703	5·16
96	0·0786	0·5110	14·14	0·7980	0·5132	14·65

Part III. Investigation of the effect of variation in the amounts of amino-acid and of sugar on the assimilation of nitrogen from amino-acids by yeast.

It has already been found (Part I) that the amount of nitrogen extracted from an amino-acid by yeast varies very considerably with the change in the concentration of the yeast cells, but, so far, the experiments have always been made with an excess of amino-acid and a large amount of sugar available for fermentation.

In the first place zymatic activity is necessary for the absorption of nitrogen by yeast, the results of a series of experiments conducted by treating amino-acid solutions with yeast in the absence of sugar proving that no nitrogen was extracted. On the other hand a further series, in which the relative rates of fermentation of sugar and decomposition of amino-acids were determined, showed that, although the one is no definite function of the other, yet the activity of the zymase does exert a stimulating effect on the desamination of the amino-acids.

A rapid fermentation, however, does not mean rapid absorption of nitrogen, for experiment shows that if fermentation is extremely rapid and consequently soon over, the desaminating enzymes apparently have not sufficient time to reach a state of activity and as a result little absorption of nitrogen takes place.

As regards the effect of the second factor mentioned above, viz., variation in the amount of amino-acid, investigation shows, as would be expected, that the quantity of nitrogen extracted is not proportional to that of amino-acid originally present, although the results obtained give some evidence that the nitrogen-content of the solution does influence the nitrogen absorption.

Worthy of note are the varying amounts of nitrogen which a yeast will assimilate under conditions apparently similar. Thus it has been observed that, with a 10 % sugar solution containing 1 % nitrogen as asparagine and seeded at the rate of 100 cells/T, the nitrogen absorbed in 24 hours has varied from 21·7 % up to 37·2 % of the amount originally present. It has, indeed, been already shown that the nitrogen content of the seed-yeast has an influence on the absorbing capacity, but in a number of cases two samples of the same yeast, after apparently identical treatment, have absorbed appreciably different amounts of nitrogen. It seems probable that such differences depend on physiological conditions of which we are as yet ignorant.

It has been found from the large number of fermentations conducted in this investigation that on an average a normal yeast seeded at the rate of 100 cells/T will extract in 24 hours 0·0250 g. of nitrogen per 100 cc. of the fermenting solution, but after the lapse of 48 hours—when the extraction will generally have reached its maximum—this amount may have increased to 0·0450 g.

Experimental.

1. *Action of living yeast on amino-acids in the absence of fermentative activity.* A washed brewery yeast was seeded into a solution of asparagine containing 0·2 % of nitrogen (1·072 g. of asparagine per 100 cc.). The liquid was subsequently maintained at 37° and at intervals of 12 hours quantities of about 75 cc. were withdrawn and filtered, the nitrogen being estimated in 50 cc. of the clear filtrate. (Schleicher and Schüll's No. 572½ filter papers are found to be especially suitable for the complete separation of yeast cells from fermented solutions.) The results thus obtained are as follows:

Table XIV.

Time in hours	0	12	24	36	48	72
Nitrogen in liquid (g. per 100 cc.)	0·2016	0·2002	0·2022	0·2038	0·2040	0·2044

The only conclusion to which these figures lead is that without fermentation no assimilation of nitrogen takes place. No importance can be attached to the apparent slight decrease of nitrogen during the first 12 hours, although later experiments seem to show that, if the yeast is in a healthy condition, assimilation may occur to a very slight extent, this probably being due to the still incomplete subsidence of the stimulus caused by a previous fermentation.

The gradual increase observed in the nitrogen-content of the solution after the initial 12 hours is of greater importance than is at first evident; this point is considered in detail in Part IV of the present paper.

2. *Relationship between nitrogen assimilation and zymatic activity.* The relative rates of assimilation of nitrogen and of fermentation were determined by means similar to those employed in the experiment described above, except that the solution here contained 10 % of cane sugar. The progress of the fermentation was followed by estimations of the sugar content of the liquid according to Bertrand's method. The results obtained are shown in Table XV.

Table XV.

Time in hours	0	6	12	24	30	36	48	60
% sugar in solution	100·00	71·00	31·00	5·00	2·75	1·20	1·20	1·20
% nitrogen in solution	100·00	—	78·00	62·80	—	49·70	42·40	42·50

Examination of these figures reveals two important points: (1) the rate at which nitrogen was assimilated in this experiment was practically constant for the first 30 hours, but subsequently underwent rapid diminution until after 50 hours absorption of nitrogen had practically ceased: (2) although fermentation of the sugar had practically ceased after 30 hours, the assimilation of nitrogen continued for 50 hours.

3. *Effect on nitrogen assimilation of rapid fermentation.* Very rapid fermentation was induced by seeding three 10 % sugar solutions containing 1 % nitrogen as asparagine with yeast at the rate of 300 (*A*), 200 (*B*), and 100 (*C*) cells/*T* respectively. After the lapse of 12 hours visible fermentation had

ceased in *A* and had nearly ceased in *B*, whilst *C* was still fermenting vigorously. At the end of 24 hours, estimations of nitrogen in the usual way gave the following results:

Table XVI.

Experiment	*A*	*B*	*C*
Rate of seeding ...	300	200	100
% nitrogen assimilated	8·50	16·80	22·60

4. *Effect on nitrogen assimilation of the amount of available nitrogen present.* The liquids employed consisted of 10 % solutions of cane sugar and a saturated solution of asparagine, these being mixed in proportions to bring the nitrogen content to 2·0, 1·0, 0·4 and 0·2 % respectively. These mixed solutions were then seeded with yeast at the rate of 100 cells/T and allowed to ferment at 37°. The fermentations were apparently complete in 24 hours, at the end of which time the amounts of nitrogen remaining in the various solutions were estimated.

The following are the results obtained:

Table XVII.

	Total nitrogen as g. per 100 cc.		
Experiment	Initial	Final	% nitrogen assimilated
A	2·0000	0·9000	55·00
B	1·0000	0·5083	49·17
C	0·4000	0·2889	27·78
D	0·2000	0·1549	22·55

Expressed as ratio, these figures are as follows:

Total nitrogen introduced in $A : B : C : D$; 100 : 50 : 20 : 10.

Total nitrogen assimilated in $A : B : C : D$; 100 : 44·7 : 10·1 : 4·1.

It must therefore be concluded that the smaller the amount of nitrogen available for extraction from the solution, the less is the power of the yeast to extract the nitrogen.

Further elucidation of this point was attempted by means of a series of experiments in which a 10 % solution of cane sugar containing 0·03, 0·04, and 0·05 g. respectively of nitrogen as leucine per 100 cc. of solution was fermented in the manner described above. Leucine was chosen here because asparagine contains both "amino" and "amido" nitrogen, and previous experiments had shown that nitrogen is not absorbed at the same rates from these two groups. It was therefore thought that with such small amounts of nitrogen present in the solutions here involved, the use of leucine would present fewer difficulties than that of asparagine. Estimations of the amounts of nitrogen present in the solutions were made at intervals of 24 hours, with the following results:

Table XVIII.

		Final nitrogen			
Experiment	Initial nitrogen	24 hours	48 hours	72 hours	96 hours
A	0·0300	0·0120	0·0030	0·0037	0·0050
B	0·0400	0·0170	0·0060	0·0045	0·0049
C	0·0500	0·0230	0·0107	0·0050	0·0045

These numbers confirm that (1) the greater the amount of available nitrogen the greater the initial rate of extraction, and (2) that, in all cases but *C*, before all the nitrogen has been absorbed, an increase in the nitrogen content of the solution is to be observed. This latter is a most important point as is shown in the final part of this paper.

Part IV. The apparent excretion of nitrogen by yeast.

Towards the end of Part III of this communication it was pointed out that experiments seemed to indicate that under certain circumstances yeast may lose nitrogenous matter, and, for want of a better term, this may be called "nitrogen excretion."

A preliminary experiment showed that a yeast of high nitrogen content (in the case in point the yeast contained 10·3 % of nitrogen on the dry matter) would, under certain conditions, lose about 35 % of its nitrogen in fermenting a pure cane sugar solution. That this excretion did not wholly consist of bodies detrimental or useless to the yeast cell was shown by the fact that these bodies could be partly reassimilated by another yeast suggesting that the action is not a true excretion. In the following pages, however, this term has been applied to this peculiar action of the yeast.

Apparently the increase in the nitrogen content of a fermenting solution, resulting from this action, may be explained in two ways. It may be the result of (1) a "normal" excretion of nitrogen by the yeast cell, or (2) a disintegration of the cells consequent upon the commencement of auto-digestion. If the action is normal then it would be thought that a high-nitrogen yeast[1] could, cell for cell, excrete more nitrogen than a starved yeast. On the other hand, if the excretion is a pathological condition, then the starved yeast should be more prone to auto-digest and consequently should release its nitrogen constituents more rapidly. Experiment showed, however, that this was not the case, but that conversely a well-nourished, high nitrogen yeast excreted more nitrogen in fermenting a certain amount of sugar than a starved yeast. Due however to the differences in the fermentative powers of yeasts under certain conditions difficulties in comparison were found as is explained in the experimental section.

The question naturally arises, "Is this action contingent on the life of the yeast cell, or only connected with the zymatic activity?" Experiment soon proved that the action was essentially a living one, for a sample of dried yeast containing very active zymase was introduced into a sugar solution in amount to make it comparable with the living yeast experiments, and it was found that between the sixth and one hundred and twentieth hours of fermentation, the liquid only gained about one-eighth the amount of nitrogen excreted by the living yeast. Another experiment in which all the enzymes had been de-

[1] It must be emphasised that in all these experiments one race of yeast was used; reference to a high-nitrogen yeast always means a well-fed yeast, containing a large percentage of nitrogen.

stroyed gave a similar result, proving that the action is essentially a vital phenomenon.

This being the case, one would naturally expect the amount of excreted nitrogen to be proportional to the number of cells present, and experiments seeded with 10, 20, 30, etc., to 150 cells per T, showed that the yeast yielded to the liquid while fermenting a 10 % cane sugar solution amounts of nitrogen proportional to the number of cells present. The initial nitrogen coefficient of the yeast was 0·0019, and the average loss per unit cell was 0·00011 in two days, and 0·00014 in five days, that is to say, the yeast excreted 5·78 % of its nitrogen in 48 hours, and 7·36 % in 120 hours.

Not only, however, is this activity dependent upon the life of the yeast, but it is also dependent upon the zymatic activity. For example, fresh yeast seeded at 100 cells/T into pure water yielded 0·41 g. N per 100 cc. in 72 hours, which amount had not perceptibly increased after 120 hours, while a similar seeding into a 10 % cane sugar solution gave 0·71 g. in 72 hours and 1·70 g. in 120 hours. There is, therefore, some connection between nitrogen excretion and fermentative activity. Before studying this connection it was necessary to consider to what extent the time factor enters into the excretory action.

A solution of invert sugar was seeded with yeast at the rate of 75 cells/T and maintained at 20° during fermentation. At intervals of time the progress of the fermentation was estimated, and at the same time the amount of nitrogen in the solution was determined. From confirmatory series of experiments it was found that the rate of fermentation of the invert sugar present was constant for about 16 to 20 hours, then rapidly decreased, until at 36 hours it had ceased. On the other hand the nitrogen excretion progressed regularly for about 75 hours, after which it rapidly decreased, and after the 110th hour the action had apparently terminated.

The constant rate of excretion was approximately, in two series A and B, 0·000419 and 0·000384 g. N per hour for 75 cells/T. Compared with the results of a previous experiment the excretion in 24 hours per unit cell would be in these two cases 0·000134 and 0·000123 as against 0·00011 previously found. Variations to this extent have constantly been found to occur, and are probably due to the exact physiological state of the initial seed yeast.

The most important conclusion to be drawn from these two experiments is that although there is no direct relation between fermentation and excretion, the activity of the zymase stimulates the latter action, and the cessation of the metabolic changes thus stimulated does not coincide with the apparent termination of the fermentative activity. As would be expected the zymatic and excretory powers of the yeast cell are two distinct functions, but the activity of the former in some way stimulates the latter into action.

This stimulatory action of fermentation was proved very clearly as follows. Water was seeded with fresh yeast at the rate of 100 cells/T, and allowed to stand for 30 hours during which time two nitrogen determinations were made. At the thirtieth hour sugar was added and nitrogen determinations

were again made on the liquid at subsequent intervals of time. No sugar was added to a second experiment and the differences in the amounts of nitrogen found in the two cases were very striking. For example after 72 hours the amount of N in the sugar solution was 1·07 g. per 100 cc. and 0·41 g. in the aqueous suspension.

Consequently nitrogen excretion is, to a certain extent, dependent on the zymatic activity of the yeast, and as the expression of the latter is the amount of fermentation, it appeared desirable to investigate the effect of the variation of the amount of sugar available. Solutions containing differing amounts of cane sugar were fermented with samples of the same yeast, and nitrogen determinations made after 24, 48, 72, and 120 hours. The results obtained show the great stimulating effect on the nitrogen excretion of increase of the amount of available sugar from 1 % to 5 %—confirmatory results being obtained for all periods of time. The stimulatory effect rapidly decreases, and above 10 % sugar increase does not result in an appreciable increased rate of nitrogen excretion.

The nitrogen thus excreted may be derived from

(1) the reserve nitrogenous food material in the yeast cell, in which case the loss of nitrogen should terminate with the exhaustion of that reserve material,

(2) the nitrogenous tissue of the cell itself and, if this is the case, it is possible that the continued action may result in the death of the organism.

It is, of course, difficult to keep a yeast in a state of prolonged fermentative activity in a solution because of the accumulation of the products of fermentation which eventually destroy the zymatic activity, but a similar state was obtained by continually transferring a yeast from one sugar solution to another as soon as fermentation finished in the first case. In this way it was found that after five such fermentations the zymatic activity of the yeast was practically destroyed, although, on introducing the exhausted yeast into a wort, after 24 hours 85 % of the cells appeared to be in good condition and 55 % of them were budding. Consequently after the fifth fermentation although exhausted the cells were not dead, and rapidly regained a normal condition. During these successive fermentations the yeast lost 37·8 % of its nitrogen, its nitrogen coefficient decreasing from 0·00200 to 0·00124. This experiment demonstrates that a yeast can lose more than a third of its nitrogen, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

All the work so far described may be said to have been concerned with yeast under abnormal conditions, the abnormality consisting in the absence of nutrient matter from the sugar solution. Much work of a fruitless character was undertaken to determine whether this excretory action takes place while the assimilatory processes are also progressing, such, *e.g.* as the tracing during fermentation of solutions containing small amounts of asparagine, leucine, alanine, etc., of the amino-index, for the index of the excreted nitrogenous

matter is not that of the amino-acid. Finally it was found that the best method was precipitation of the fermented solution by phosphotungstic acid, for 35–37 % of the excreted matter is precipitated by this reagent. The method employed consisted of the fermentation of sugar solutions containing non-precipitable nitrogenous matter, with subsequent estimation at intervals of the nitrogen precipitated by the reagent.

The results conclusively proved that even when rapid assimilation of nitrogen was taking place, excreted nitrogen could also be found in the solution, although the absolute amount under different conditions was subject to wide variation, the cause of which was not determined.

Experimental.

1. *Preliminary experiment.* A 20 % cane sugar solution was seeded with yeast containing a high percentage of nitrogen, at the rate of $100/T$, and during fermentation carried out at 10° (in order to obtain a slower action) the nitrogen in solution was determined every 24 hours. The yeast employed was a sample of brewer's yeast and from Table XIX it will be seen that during its fermentation (which, judged by the settlement of the yeast, lasted about seven days) it lost a very considerable amount of nitrogen to the liquid.

Table XIX *a*.

Time in hours	24	48	72	96	120	240
N in liquid as g. per 100 cc.	0·0760	0·0840	0·1520	—	0·2440	0·3360

Table XIX *b*.

	Total N in solution	Total N in yeast	% N in yeast on dry matter	% N lost by yeast
Initial	Nil	1·3520	10·30	—
Final	0·3360	1·0160	7·74	24·85

2. *Assimilation by yeast of nitrogenous matter previously excreted.* After the fifth day of fermentation, 250 cc. of the solution was extracted, filtered and vacuum distilled at 40°. In this way the residue was obtained free from alcohol and water at a temperature at which practically no change would have taken place in the constitution of the amino-acids. The residue so obtained was dissolved in 10 % sugar solution and fermented with a normal yeast in the ordinary manner. The yeast contained 8·2 % nitrogen on the dry matter; nitrogen determinations made on the solution gave the following results:

Table XX.

Time in hours	0	48	120
Total N in solution	0·0620	0·0240	0·0370

The fermentation was practically over at the end of the second day when 61·30 % of the original nitrogen had been reassimilated by the yeast, and it is interesting to note that at the end of five days a further excretion had taken

place. This suggests that about one-third of the original excretion is not assimilable by the yeast, which may be that portion precipitable by phosphotungstic acid.

3. *Comparison of the excretory powers of yeast of high and low nitrogen coefficient.* Sample *A* was a well-nourished yeast containing 9·2 % nitrogen on the dry matter, while *B* only contained 7·0 % of nitrogen. The yeast was introduced at the rate of 100 cells/*T* into 10 % sugar solution and fermentation allowed to proceed at 37° for four days, during which time determinations of the nitrogen content of the solution were made after 48 hours and at the termination of the experiment.

Table XXI.

Yeast condition	% N in yeast on dry matter	N in liquid	
		48 hours	96 hours
High nitrogen	9·2	0·3052	0·3648
Low nitrogen	7·0	0·2027	0·3500

The starved yeast was sluggish of action and did not finish fermentation until the expiration of four days, the well-fed yeast had completely finished in less than 48 hours. The figures show the stimulatory effect of fermentation on the excretion of nitrogen, an effect which is more fully studied in a later experiment.

4. *Comparison of living and air-dried yeast as regards nitrogen excretion.* A yeast which had been air dried at 37°, and afterwards ground up in a mortar, was used, the preparation having been made some 12 months before use. Sufficient was added to a 10 % sugar solution to bring the nitrogen content of the solution up to 0·1352 N per 100 cc., thus making comparison with the experiment previously described possible. The mixture was allowed to stand at 37° and a vigorous fermentation took place, showing that the zymase was still in a state of activity. After periods of 6 and 120 hours the nitrogen content of the solution was measured, the amount of nitrogen introduced into the solution mechanically being obtained from the first reading.

Table XXII.

Experiment	N in solution g. per litre	
	6 hours	120 hours
Dried yeast	0·0304	0·0568
Living yeast	0·0150	0·2440

In the case of the dried yeast it is obvious that 0·0304 g., or nearly 60 % of the total amount of nitrogen given to the solution, was introduced mechanically in a soluble form, while it is to be noted that in 120 hours the *total* amount given to the liquid is less than 25 % of that excreted by the living cell.

5. *Comparison of living yeast with one in which all the enzymes had been destroyed.* The experiment was conducted on exactly parallel lines, except that yeast previously dried at 95° was used, and gave the results set out in the following table, in which the figures for living yeast are shown for purposes of comparison.

Table XXIII.

Experiment	N in solution g. per litre 6 hours	120 hours
Dried at 95°	0·0535	0·0862
Living yeast	0·0150	0·2440

6. *Effect of varying the amount of yeast present.* A series of experiments was made in which 10 % sugar solution was seeded with yeast at the rate of 10, 20, 40, 80, 150, and 320 cells/T. On plotting the results it became apparent that as far as this series went, the excretion varied in a regular manner with the amount of yeast present. The latter points, however, were so far apart in comparison with the first three, that the experiments were repeated in an extended form, 15 fermentations being carried out, the seeding being from 10 to 150 cells per unit volume. Nitrogen determinations were made at the end of 48 and 120 hours, those in the lower seedings being made on amounts of liquid varying up to 250 cc. The results—Table XXIV—are given as grams of nitrogen per litre.

Table XXIV.

Rate of seeding ...	10	20	30	40	50	60	70	80
Nitrogen in solution after 48 hours ...	—	0·021	—	0·048	0·054	0·064	0·076	0·122
Nitrogen in solution after 120 hours ...	0·025	0·024	0·050	0·055	0·068	0·081	0·104	0·110
Rate of seeding ...	90	100	110	120	130	140	150	
Nitrogen in solution after 48 hours ...	—	—	0·116	0·132	0·136	—	0·144	
Nitrogen in solution after 120 hours ...	0·131	0·129	0·144	0·172	0·208	0·192	0·221	

The results are expressed graphically in Fig. 1, and it must be remembered on examining these figures that the vertical scale is a very large one, so that, excluding the 80 cell experiment in the second day, the greatest error occurs in the 120 cell experiment, and is equal to 0·00084 g. N per 100 cc.

The curves show without doubt that the amount of nitrogen excreted is proportional to the amount of yeast introduced into the sugar solution, other factors being constant.

The following table shows, in brief, the loss of nitrogen by the yeast.

Table XXV.

Time	Nitrogen coefficient × 10^4 Initial	Final	% N lost by yeast
48 hours	19·0	17·9	5·78
120 hours	19·0	17·6	7·36

7. *Effect of the time factor in nitrogen excretion.* A solution of invert sugar containing 10 g. per 100 cc. was seeded with yeast at the rate of 75 cells/T and allowed to ferment at 20°. For the first two days the amount of sugar fermented and the nitrogen excreted were determined every two hours from

8 a.m. to 8 p.m. and afterwards with varying intervals of time for five days[1]. The following are the results of two experiments:

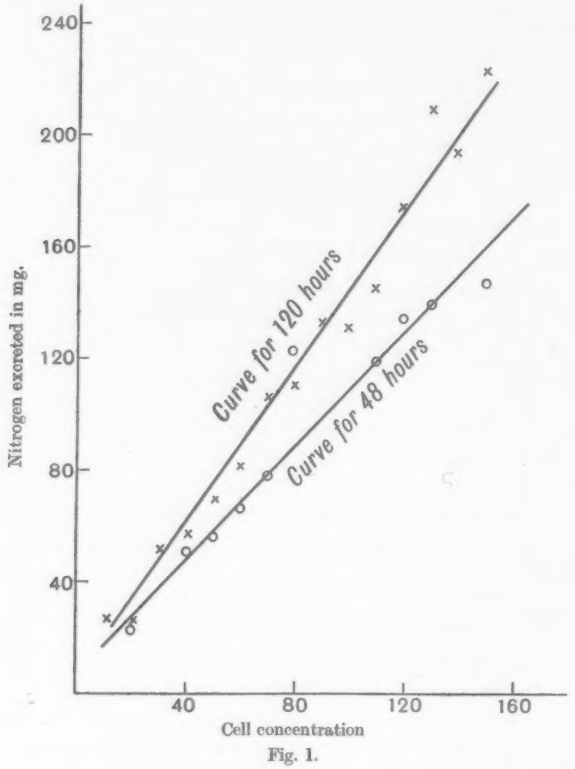


Fig. 1.

Table XXVI.

Experiment A.

Time in hours	2	4	6	8	10	12	24	26	28	30	32	34
Sugar fermented	1·70	1·80	2·90	4·60	5·50	6·90	8·70	9·50	9·62	9·73	9·85	9·87
N excreted*	—	0·90	0·74	0·76	1·26	0·84	1·18	—	1·28	—	1·34	1·30

Time in hours	36	48	52	56	60	72	78	84	96	102	120	132
Sugar fermented	9·88	9·90	.....				Constant					.....
N excreted*	1·56	1·66	1·70	1·64	1·94	2·14	2·12	2·12	2·02	2·20	2·22	2·26

* As g. $\times 10^2$ per 100 cc.

[1] The invert was prepared from cane sugar by taking a 40 % solution and hydrolysing in a steam steriliser for three hours with 5 cc. of strong hydrochloric acid per litre of solution. The optical rotation was then found to be constant, the acid was neutralised with sodium hydrate, and the solution diluted until it contained 10 % invert sugar. The determinations of the invert were made according to the method of Bertrand (*Manipulations de Chemie Biologique*).

Table XXVII.

Experiment B.

Time in hours	12	14	16	18	20	22	24	36	40		
% sugar fermented ...	55·5	60·5	74·5	—	85·0	87·0	93·8	98·7	98·8		
N excreted*	0·98	1·04	0·88	—	—	1·20	1·24	1·70	—		
Time in hours	44	48	60	66	72	84	90	96	108	120	
% sugar fermented ...	98·9	99·3	.....	.....	.....	Constant	.....	.....	.....	.....	
N excreted*	—	1·90	1·94	2·06	2·42	—	2·72	2·82	3·32	2·96	

* As g. $\times 10^2$ per 100 cc.

From these two sets of experiments (see Fig. 2 for Exp. *A*), which confirm each other, it will be seen that the fermentation proceeds regularly for about 16 hours in the first case, and for about 20 hours in the second; after this period the rate decreases and practically all the fermentable matter has been removed in 36 hours in both cases. The excretion of nitrogen, however, proceeds regularly for nearly 70 hours in Experiment *A* and for 84 hours in Experiment *B*, after which its rate rapidly decreases and another stage is apparently reached after the 110th hour.

8. *Effect of zymatic activity on nitrogen excretion.* Water was seeded with fresh yeast at the rate of 100 cells/*T* and allowed to stand for 30 hours, during which time two nitrogen determinations were made. At the thirtieth hour fermentation was induced in one portion by the addition of cane sugar at the rate of 10 g. per 100 cc. of solution (Exp. *A*) while the other was left as a mere suspension (Exp. *B*). Nitrogen determinations after successive intervals of time gave the figures shown in the following table, which clearly prove the great stimulatory effect of fermentative activity. The somewhat large amount of nitrogen given to the solution by the yeast alone may be attributed to its fresh and healthy condition when first introduced into the solution. The results are expressed graphically in Fig. 3.

Table XXVIII.

Time in hours	24	30	48	54	72	96	120	
Exp. *A* } N excreted as	0·16	0·21	0·55	0·48	1·07	1·44	1·70	
Exp. *B* } g. $\times 10^2$ per 100 cc.	0·16	0·21	0·28	0·34	0·41	—	—	

9. *Effect on nitrogen excretion of available sugar.* Fermentation may be regarded as a function of the sugar present, and consequently the sugar factor appeared to be an important one. Solutions containing 1·0, 2·0, 3·0, 4·0, 5·0, 10·0, 20·0, and 40·0 % of sugar were subjected to the action of yeast, seeded at the rate of 100 cells/*T*, the fermentation being allowed to proceed at 20°. After 24, 48, 72 and 120 hours the nitrogen in the liquid was determined, with the results given in Table XXIX.

Table XXIX.

Sugar content %		1	2	3	4	5	10	20	40
N excreted as g. $\times 10^2$ per 100 cc.	After 24 hrs.	0·20	—	0·28	0·28	0·27	0·30	0·33	0·36
	,, 48 ,,	0·26	—	0·30	0·28	0·37	0·36	0·40	0·44
	,, 72 ,,	0·27	0·30	—	0·34	0·35	0·42	—	0·52
	,, 120 ,,	0·28	0·35	0·38	—	0·40	—	0·53	0·58

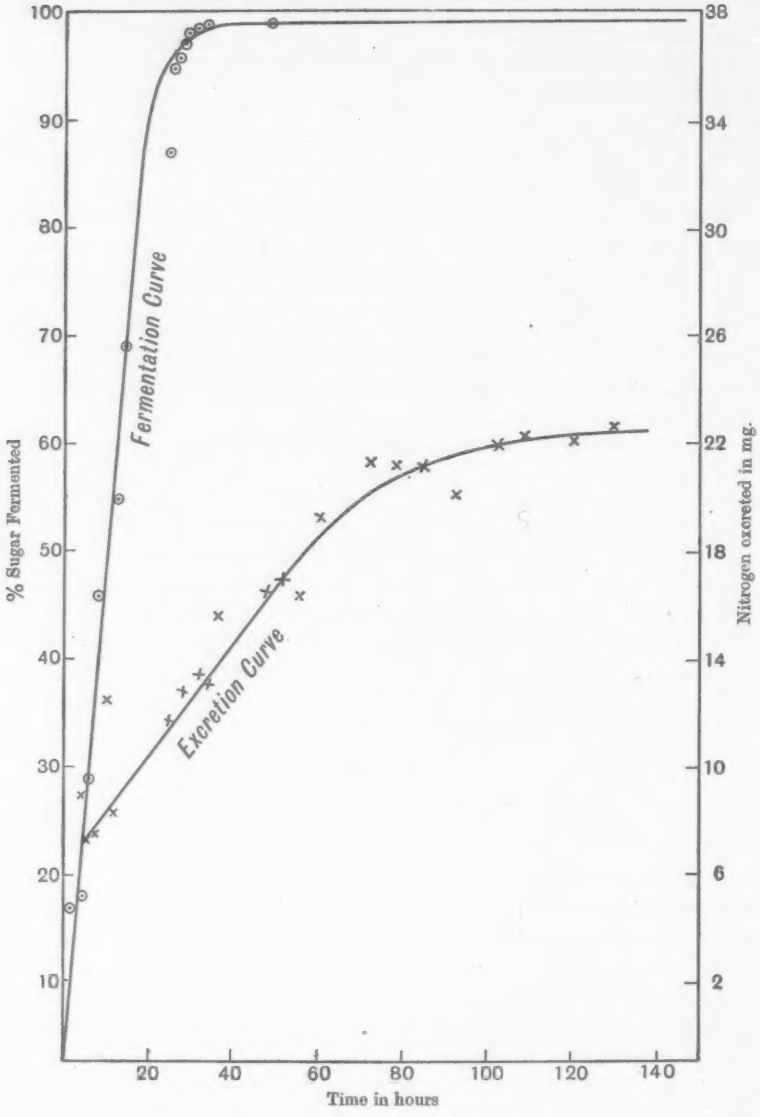

100
90
80
70
60
50
40
30
20
10
% Sugar Fermented
38
34
30
26
22
18
14
10
6
2
Nitrogen excreted in mg.
Fermentation Curve
Excretion Curve
20
40
60
80
100
120
140
Time in hours

Fig. 2

These figures have already been studied in the theoretical part of the paper, and it only remains to be said that graphically they demonstrate very clearly the conclusions drawn.

10. *Effect of exhausting the fermentative powers of the yeast.* A litre of 20 % cane sugar solution was seeded with yeast—nitrogen coefficient 0·0020—at the rate of 100 cells/T, and the fermentation allowed to proceed at 37°. At the conclusion of the fermentation—about 32 hours—the yeast was washed once

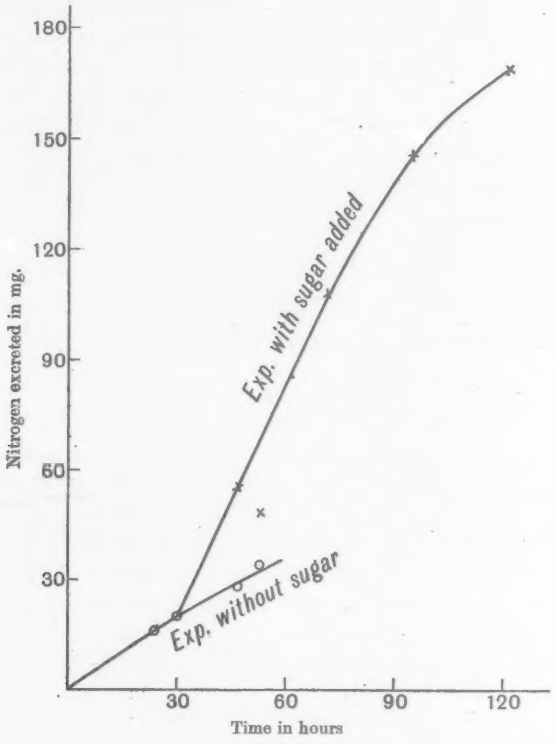


Fig. 3.

by decantation and filtered off through a Buchner funnel. The liquid, which was not quite clear, was treated with alumina cream, refiltered, and the perfectly bright filtrate acidulated with 100 cc. $N/20$ sulphuric acid (to prevent the escape of any ammonia) and concentrated to 200 cc. The nitrogen was determined in an aliquot portion of this solution.

The previously separated yeast was introduced into a further litre of 20 % sugar solution and allowed to ferment at 37°. The process of separation was carried out as described above, at the termination of the fermentation and the

whole process repeated. After the third fermentation the yeast had assumed an appearance altogether different from its original condition, for, although the cells looked perfectly healthy and approximately 5 % of them were budding, the mass was of a slimy, sticky nature, extremely difficult to filter. This appearance was much more marked after the fourth fermentation, which had been of a sluggish nature, and had taken much longer to commence, and when the yeast was introduced into the sugar solution for the fifth time the action was extremely slow. The yeast separated from this fermentation had apparently lost its zymatic power, but on being introduced into a wort (gravity 1053) it was found that after 24 hours about 85 % of the cells seemed to be in a fairly good condition and about 55 % of them were budding.

The revivified yeast was again sown into wort, and after a further 24 hours only a very few poor cells were to be seen, and the majority were budding, thus demonstrating that although after the fifth fermentation the yeast was very exhausted, the cells were not dead. The following figures show the amounts of nitrogen lost during the successive fermentations:

Table XXX.

No. of fermentation	1	2	3	4	5
N excreted during fermentation	0·1160	0·1554	0·1967	0·1596	0·1293

Total amount of N excreted	0·7570
Total N originally in the yeast	2·0000 g.
% N lost during five fermentations	37·85

There is apparently no explanation of the irregularity of the amounts of nitrogen excreted during different intervals, unless it be, and this is perhaps an important factor, that the aeration of the cells between the successive fermentations varied in amount and in efficiency; this might easily result from the varying states of the yeast and consequently the slightly different methods of filtration employed. However, the experiment demonstrates that during fermentation the yeast has excreted 37·8 % of its nitrogen, and not only this, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

11. *Determination of excretory matter in the presence of amino-acids.* Leucine was added in varying amounts to 20 % cane sugar solutions and fermented with yeast introduced at the rates of 50 and 100 cells/T; controls were made in which no yeast was added in one case and no sugar in another. At intervals the nitrogen was estimated as usual, and at the same time 50 cc. of the clear filtrate treated with 5 cc. phosphotungstic acid solution (10 % phosphotungstic acid in 5 % sulphuric acid). The mixture was stirred and the precipitate which formed allowed to settle, which it did very well in about 12 hours. The supernatant liquid was then decanted off and the precipitate—which in character was of a rather heavy, flocculent nature, and of an almost white or pale clay colour—mixed with about 15–20 cc. of the precipitant [Brown, 1903,

2] and filtered through an ordinary filter paper of a fairly loose texture[1]; nitrogen determinations were subsequently made on the clear filtrate. Table XXXI shows the figures obtained for one such series.

Table XXXI.

Exp.	% sugar	Initial N as amino-acid	Final N after 48 hours	N pptd by phospho-tungstic acid	Change in N content of solution
A	0	0	0·0052	0·0020	+0·0052
B	0	0·0584	0·0600	Trace	+0·0016
C	20	0	0·0528	0·0200	+0·0528
D	20	0·0584	0·0308	0·0232	−0·0276
E	20	0·0630	0·0500	0·0232	−0·0130
F	20	0·1168	0·0552	0·0306	−0·0616

These figures show that:

1. Where fermentation has not occurred, no precipitable nitrogen, or practically none, appears in the solution.

2. The greater the amount of nitrogen assimilated, the greater the amount of precipitable nitrogen found.

General Conclusions.

1. *Factors which influence the assimilation of nitrogen by yeast.*

(*a*) Excess of yeast ensures the removal of the greatest *total* amount of nitrogen.

(*b*) During active fermentation the greater the coefficient of multiplication, the greater the amount of nitrogen assimilated by *each* cell.

(*c*) Active reproduction may result in a lowering of the nitrogen coefficient, even when a large amount of available nitrogen is present.

(*d*) The final nitrogen coefficient of a yeast is independent of the initial coefficient, and tends to reach a value constant for any particular conditions of reproduction.

2. *Mechanism of the extraction of nitrogen from amino-acids by yeast.*

(*a*) The amidase of Effront does not (as stated by him) produce solely a volatile acid by its action on asparagine, but also a non-volatile acid, probably malic acid.

(*b*) If malic acid represents the first stage in the degradation of asparagine, it is not fermented as such, for the acid is unattacked by yeast. It may combine with NH_3 to form the ammonium salt which is completely destroyed by fermenting yeast, ethyl alcohol being the only recognised product of the action.

[1] The first runnings had always to be returned, but after a few minutes the filtrate came through quite bright. The clear liquid was warmed to about 40° and treated with ground baryta with constant stirring, in order to remove the excess of phosphotungstic and sulphuric acids. A slight excess only of the baryta was added, and the liquid allowed to settle, the clear liquid decanted off, and the remainder filtered and the precipitate washed. The excess of baryta was then removed by carbon dioxide, and the nitrogen estimated in the filtrate. It will be understood that in face of the number of processes extreme care was required to obtain concordant nitrogen readings.

(*c*) Propionic acid (which Effront states results from the action of amidase on asparagine) is not fermentable, and its ammonium salt is attacked with difficulty by yeast.

3. *The influence of the available amino-acids and sugar on the nitrogen assimilation by yeast.*

(*a*) Fermentative activity is essential to nitrogen assimilation. The two actions are not proportionate, but the former stimulates the latter, and, once induced, desamination may continue after zymatic activity ceases.

(*b*) Excessive zymatic activity does not ensure rapid nitrogen assimilation, in fact a quick fermentation results in only a small amount of nitrogen being extracted.

4. *Apparent excretion of nitrogen by yeast.*

(*a*) During fermentation yeast continually loses nitrogen to the liquid, and this action has been called "nitrogen excretion." Cases have been noted where more than 33 % of the yeast nitrogen was so lost, but further loss resulted in the disablement of the cell.

(*b*) Nitrogen excretion is dependent on the *life* of the cell, and takes place even when nitrogen is being assimilated.

(*c*) The bodies so excreted can be used by yeast as a source of nitrogen under suitable conditions.

(*d*) Increase in the amount of sugar available for fermentation increases the rate of excretion, especially between the limits 1–5 % sugar, and although zymatic activity is necessary for nitrogen excretion, the two are not proportionate, nor does the cessation of the former result in an immediate cessation of the latter.

The Author desires to acknowledge his great indebtedness to the late Professor Adrian J. Brown, F.R.S., for the kindly advice and sympathy extended to him during the course of the above research (which was concluded prior to 1914); to Mr T. H. Pope, B.Sc., for his keen interest in the work, and to Professor A. Harden, F.R.S., for his great assistance in publication.

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XLI. THE RELATIONSHIP OF LECITHIN TO THE GROWTH CYCLE IN CRUSTACEA.

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The period leading up to moulting in Crustacea is characterised by certain metabolic and anatomical changes, some of which we have dealt with in previous papers [Paul, 1914; Paul and Sharpe, 1916]. Briefly, these are as follows. There is a marked increase of fat, glycogen, and calcium in the liver, the last reaching as much as 20 % of the dried weight of the organ. At the same time mitosis occurs rapidly in the cells of the integument and other tissues of the body, but no increase in the size of the animal takes place till the rigid shell is cast and rapid absorption of water makes the animal increase in size.

When the moult is complete, an exodus of stored material takes place from the liver; the fat is broken down to glycerol and lower fatty acids before it enters the blood, and calcium is carried off as soaps of lower fatty acids to be deposited in the integument as calcium carbonate. Cell proliferation in the body then ceases, but increase in the size of the cells is marked.

After a period of rest the crab again stores material in the liver in preparation for the next moult. It has been found that as the period of moulting approaches the liver increases in weight relatively to the body. We have therefore employed the term *hepatic factor* to denote this relationship, and have applied it to the figure obtained by dividing body weight by weight of fresh liver. Shortly after moulting this factor may be 10 or 12, but as the storage goes on it may fall to five or even lower [Paul, 1915]. We therefore use the hepatic factor to indicate proximity to moulting.

Fat is present in the liver in quantities varying from 20 to 30 % of the total gland. In this fat we found phospholipins. The question arose as to the relationship of these substances to the metabolic cycle, and we have examined many individuals of the Decapod Crustacea to determine what it is.

The results of four of these examinations carried out on crabs (*Cancer pagurus*) at different periods between the moults are to be found in the table and graph hereunder.

Methods.

The legs were removed by the production of autotomy [Paul, 1915] at the breaking-planes, and the weight of the body was then taken. The fresh liver was removed and put into ether-alcohol mixture (50 %). The fats were extracted by repeated washings with ether and hot alcohol and weighed. The fat was then ashed and the total amount of phosphorus present was determined by the ordinary molybdate method. Using the formula $C_{43}H_{85}O_8NPH$ [MacLean, 1918] this phosphorus was calculated to lecithin.

We consider the above method more exact than that in which acetone is employed to precipitate the phospholipins as such, and the calculation has been made to *true lecithin* [MacLean, 1918] in all cases, for purposes of comparison only.

Results.

We find that the amount of fat in the liver increases both in total quantity and relatively to the weight of the body and liver as the animal approaches the moult.

It is also made clear that the amount of phospholipin in the liver (calculated to lecithin) diminishes both in total quantity and relatively to the weight of the body and liver as the animal approaches the moult.

Conclusions.

The relationship of phospholipin metabolism to developmental changes has been the subject of varied researches.

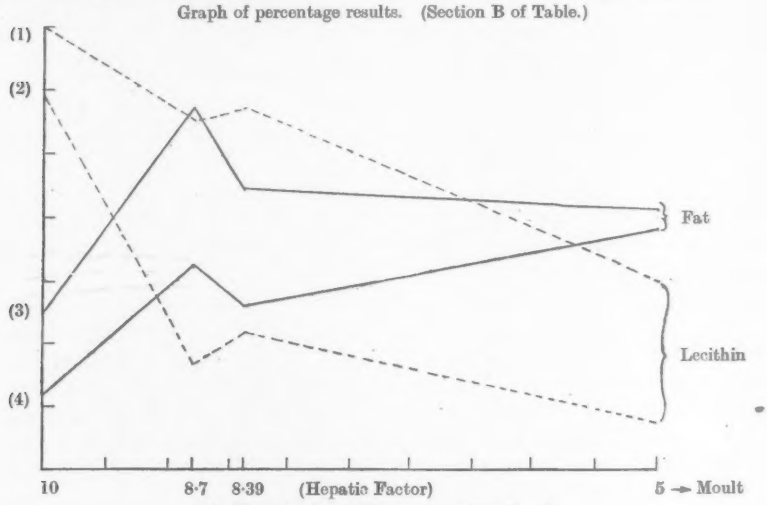
Noël Paton has shown that lecithin accumulates in the ovary of the salmon at the expense of the inorganic phosphorus of the muscle [1898]. The same author has indicated the increased percentage of lecithin in liver fats of mammals during starvation, and has associated it with the storage of phosphorus for nuclein formation [1896]. Hoppe-Seyler has demonstrated the high lecithin content of embryonic tissues [1887] and Plimmer and Scott have indicated the marked diminution of lecithin in hens' eggs during development of the embryo [1909]. More recently Brailsford Robertson, studying the development of certain sea-urchin eggs, has shown that there is a marked decrease of lecithin whilst nucleoprotein is increasing in the egg [Robertson and Wasteneys, 1913]. The same author, working with Burnett, has also described decrease in the rate of growth of carcinoma in rats, which he ascribes to the injection of lecithin into the tumor [1913]. Again, Brailsford Robertson has indicated by experiments on developing echinoderm eggs that lecithin is the possible auto-catalyst of growth [1913] and Halliburton [1909] has pointed out that the formation of choline or choline soaps by the splitting up of lecithin or other phospholipins may cause that alteration of surface tension around the nucleus which is the prelude to division.

The present work therefore falls into line with previous researches. It shows that the phospholipin of crustacean livers decreases as cell proliferation advances, and the nuclei, though not the cell bodies, become larger. Since the integument is composed of calcium carbonate, the phosphorus cannot be associated with integument formation, comparable to calcium phosphate deposition in vertebrate bone. It is strongly probable, therefore, that at least some, if not all, of the phosphorus of the nucleoprotein molecule is derived from the phospholipin molecule.

	A				B				
Crab	Weight without legs	Weight of fresh liver	Fat in liver	Phospho-lipin as lecithin	Liver fat % of crab	Fat % of liver	Lecithin % of crab	Lecithin % of liver	Hepatic factor
1	283	53	10·95	0·077	3·8	20·6	0·03	0·7	5+
2	382	45·5	10·07	0·2179	2·6	22·1	0·057	2·1	8·39
3	339	38·7	11·01	0·1892	3·2	28·4	0·055	1·7	8·7
4	283	28·4	3·53	0·2091	1·2	12·4	0·07	5·9	10−

A = Quantitative results in grams.
B = Percentage results. These are graphically represented in relation to hepatic variation.

Graph of percentage results. (Section B of Table.)



(1) Hepatic phospholipin percentage of crab.
(2) Phospholipin percentage of fresh liver.
(3) Fat percentage of fresh liver.
(4) Hepatic fat percentage of crab.

On the Y-axis (1) Five small divisions = 0·01 %.
(2) „ „ = 1 %.
(3) „ „ = 5 %.
(4) „ „ = 1 %.

We have pleasure in expressing indebtedness to Professor Noël Paton for his encouragement in the work, and for his sound advice on such parts of it as were carried out in the Physiological Department of Glasgow University.

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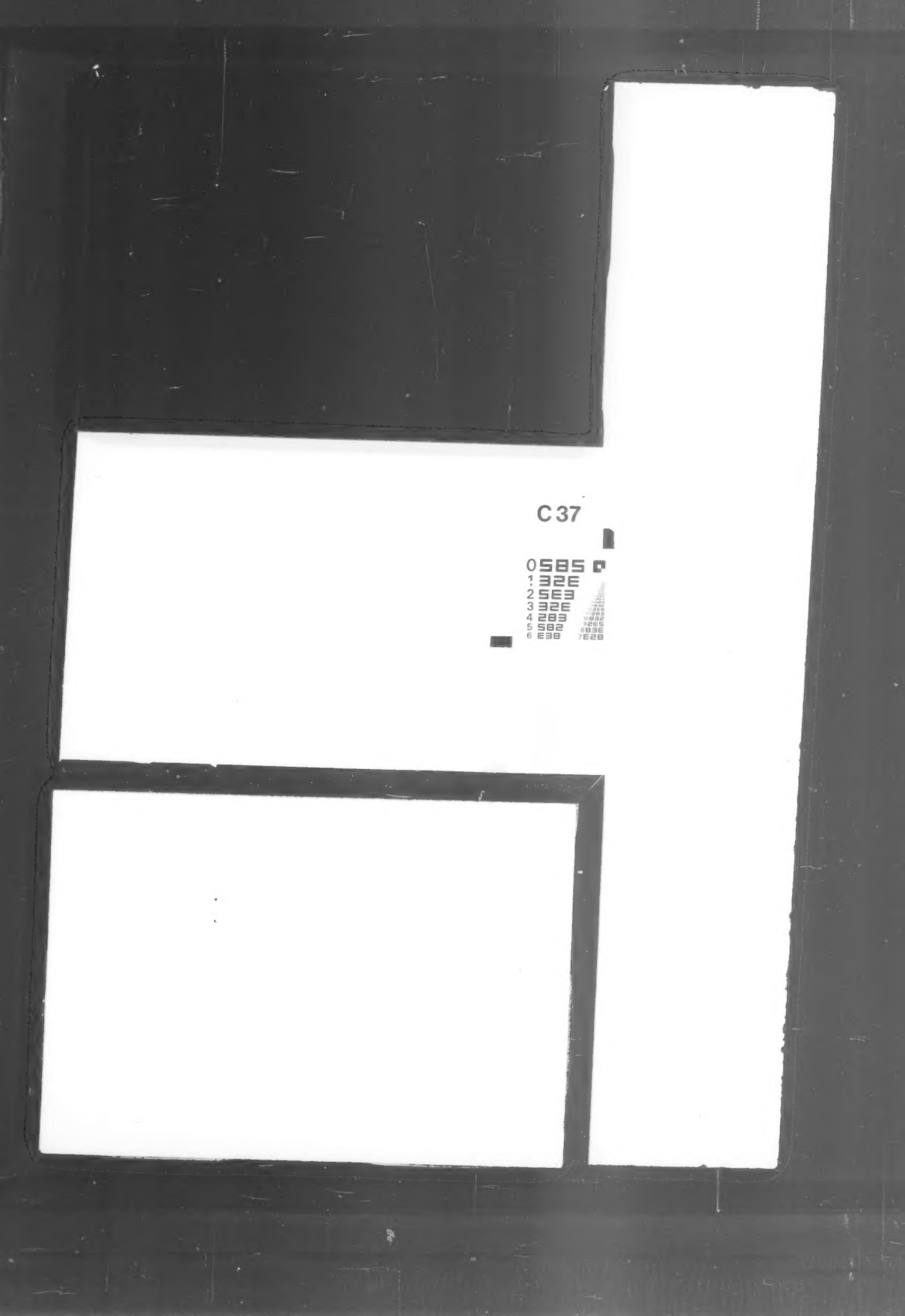
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